

Effect of phytochemical-rich foods on *in vivo* NF-κB activity in transgenic reporter mice

Master Thesis by

Marit Kolby Pedersen



Supervisors:

Rune Blomhoff

Ingvild Paur

Trude Rakel Balstad

Department of Nutrition, Faculty of Medicine

UNIVERSITY of OSLO

May 2009

Acknowledgements

I have worked on this thesis from August 2008 to May 2009, at the Department of Nutrition, Faculty of Medicine, University of Oslo.

I would like to thank my supervisor Rune Blomhoff for being such an inspiring advocate of nutritional science and for taking the time to critically review my work.

Ingvild Paur and Trude Rakel Balstad – you are the most encouraging, dedicated, patient and cool supervisors any student could ask for. You always take the time to teach and guide and you have included me in your work in a way that is much appreciated.

Thanks to everyone in Rune Blomhoff's research group for sharing your knowledge and for creating an inspiring environment. Thanks to Kari Holte for running the FRAP analysis on my extracts.

A great, big thank you to Hanne, you are a great friend, a helpful and sharing collaborator and an excellent partner for recreational running in Nordmarka.

Thanks to my family and friends for your support during my education.

Elin - thank you for proofreading my manuscript.

Finally, Severin - your support, advice and simply your presence is invaluable to me.

Summary

The connection between a high intake of dietary plants and a reduced risk of degenerative diseases is supported by a large amount of epidemiological evidence. Emerging evidence from nutritional genomics suggest that plants exert their protective effect through affecting cellular signalling and gene expression. Aberrant NF- κ B activation has been linked to various diseases, and several phytochemicals possess NF- κ B modulating properties that could have potential in prevention of disease. In this thesis, the modulation of NF- κ B activation by phytochemical-rich plant extracts was studied by the use of transgenic reporter mice.

The aims of this project were to perform *in vivo* experiments with an extract combined of five efficient *in vitro* NF- κ B inhibitors. Secondly, the thesis involved separate studies on the *in vivo* NF- κ B modulating potential of three of the five dietary plants found in the combination extract.

The treatment of transgenic reporter mice with combination extract significantly inhibited lipopolysaccharide (LPS)-induced NF- κ B activity. The area under curve (AUC) was 35 % lower in the extract group compared to the control group (0-6 h). We found organ specific inhibition of NF- κ B, particularly in the organs of phytochemical metabolism, as well as in the male reproductive organs.

The *in vivo* experiments with thyme, clove and walnut extracts revealed a significant difference of 104% higher overall NF- κ B activation at 6 h in the walnut group compared to the control group. We also found organ specific NF- κ B modulation by the single food extracts, particularly in the liver, spleen and the male reproductive organs

We have found that food extracts can modulate LPS-induced NF- κ B activation, on an overall basis, and in an organ-specific manner. Based on this thesis, further work to elucidate the mechanisms of action of the NF- κ B modulating food extracts is a possible future area of priority.

Contents

ACKNOWLEDGEMENTS.....	3
SUMMARY	5
CONTENTS.....	6
LIST OF ABBREVIATIONS	8
1. INTRODUCTION	9
1.1 NUTRITIONAL SCIENCE APPROACHES.....	9
1.2 DIETARY PLANTS AND HEALTH.....	11
1.3 PHYTOCHEMICALS.....	12
1.4 PHYTOCHEMICALS AND DIET	16
1.5 OXIDATIVE STRESS AND ANTIOXIDANT DEFENSE	18
1.6 THE NF- κ B FAMILY OF TRANSCRIPTION FACTORS.....	22
2. AIM OF THE THESIS.....	30
3. MATERIALS.....	31
o TRANSGENIC MICE	31
o TRANSGENIC MICE EQUIPMENT.....	31
o CHEMICALS	31
o DIETARY PLANT EXTRACTS.....	32
o KITS	32
o INSTRUMENTS.....	33
o SOFTWARE	33
4. METHODS.....	34
4.1 DIETARY PLANTS.....	34

4.2	EXTRACT PREPARATION	34
4.3	FRAP ASSAY ANALYSIS	35
4.4	LUCIFERASE REPORTER SYSTEM	36
4.5	<i>IN VIVO</i> EXPERIMENTS	37
4.6	STATISTICAL ANALYSIS	46
5.	RESULTS	47
5.1	FRAP ANALYSIS.....	47
5.2	<i>IN VIVO</i> EXPERIMENTS	47
6.	DISCUSSION.....	62
6.1	DISCUSSION OF METHODS.....	62
6.2	GENERAL DISCUSSION	66
7.	CONCLUSION	77
8.	REFERENCE LIST.....	78
9.	APPENDIX	84

List of abbreviations

AUC	Area under curve
CAT	Catalase
CCl ₄	Carbon tetrachloride
ddH ₂ O	Double distilled water
DIM	3,3-Diindolylmethane
EGCG	Epigallocatechingallate
FRAP	Ferric reducing ability of plasma
GPx	Glutathione peroxidase
HSP	Heat shock protein
HPLC	High performance liquid chromatography
IKK	IκB kinase
IκB	Inhibitor of nuclear factor κB
LUC	Luciferase
LPS	Lipopolysaccharide
NF-κB	Nuclear factor kappa B
NLS	Nuclear localisation signal
Nrf2	Nuclear factor erythroid2-related factor
NEMO	NF-κB essential modulator
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PUFA	Poly-unsaturated fatty acid
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RLU	Relative luminescence unit
RHD	Rel homology domain
SOD	Superoxide dismutase
TLR	Toll like receptor
TNFα	Tumour-necrosis factor-α
TPTZ	Tripyrdyltriazine

1. Introduction

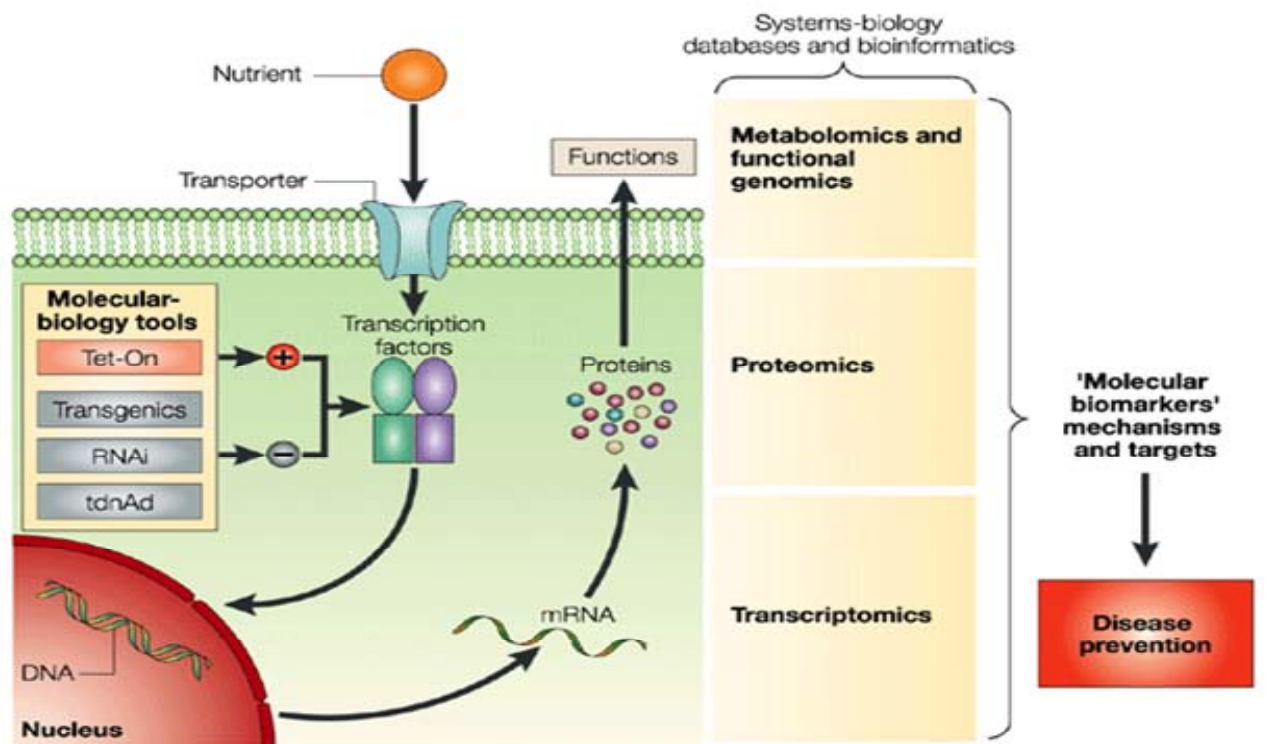
1.1 Nutritional science approaches

Traditional epidemiology and physiology, though still fundamental to nutritional science, have been complemented by a notable focus on genetics and molecular biology, particularly during the last two decades. There has been a growing awareness among nutritional scientists that, to be fully understood, nutrition's role in disease prevention and health should be investigated at the molecular level. According to Müller and Kersten, there are three main reasons for the expanded focus in nutritional scientific approach (1):

- Completion of large genome projects has provided a multitude of genetic information, making it possible to study nutrition from this specific point of view.
- The fact that compounds in food, such as micronutrients, macronutrients and phytochemicals can act as dietary signals influencing the metabolism has been recognised and implemented in research approaches.
- The importance of genetic predisposition in the context of diet related diseases has been acknowledged.

This field of study is referred to as nutritional genomics, or nutrigenomics, and aims at revealing the genome wide influence of nutrition on homeostasis. Nutrigenomics are investigated by two different approaches, providing complimentary information.

Figure 1.1 presents a schematic overview (1).



Nature Reviews | Genetics

Figure 1.1. The combination of methods in nutrigenomics. Molecular tools can be used to discover dietary target genes and the mechanisms involved in their regulation (left), and systems biology can identify molecular biomarkers, as well as target genes and mechanisms. Dietary advice can prevent disease (right). From Müller and Kersten (1).

The first approach is the use of for example cellular models and transgenic mice to study genes and the proteins they code for, their regulatory pathways and how they are affected by dietary factors (1). The second is systems biology, which is still in its scientific infancy, but is given huge expectations for future research. Systems biology is the study of complex interactions in a biological system and the dynamic relationships between biological molecules, as opposed to the classical reductionist approach in science. Nutritional systems biology will combine data on the transcriptome, proteome and metabolome to identify molecular biomarkers, targets and mechanisms involved in the interplay between diet and health (1).

The two approaches combined can provide a better understanding of biological dysregulation that leads to diet-related diseases, and to identify genotypes that bear a larger risk of developing these diseases (1). Such knowledge could lead to adjustment of dietary recommendations on basis of genetic information, a possibly valuable tool for composing personalized diets for people with a genetic susceptibility to develop a

specific disease. While tailor-made diets might be a future scenario in nutrition, there is a great potential of improvement of public health by influencing dietary habits at a general population level.

1.2 Dietary plants and health

The connection between a high intake of dietary plants and a lower risk of degenerative diseases such as cardiovascular disease, several types of cancer and diabetes, is supported by a significant amount of epidemiological evidence. The World Health Report 2003 from the World Health Organisation (WHO) states these facts about the consumption of fruits and vegetables and health (2):

- Insufficient intake of fruits and vegetables is estimated to cause 19% of gastrointestinal cancer, 31% of ischaemic heart disease and 11% of stroke on a global basis.
- Low fruit and vegetable intake is among the top 10 selected risk factors for mortality.
- Sufficient intake of fruit and vegetables could prevent 2.7 million deaths annually.

The association between health and diets rich in plant based foods has been studied for many years and by many strategies, molecular as well as epidemiological. The positive effects of e.g. vitamins, minerals and dietary fibre are well established, but new insight in molecular pathways of dietary components is adding new levels to the significance of diet. Emerging evidence suggest that the protective effects of plants also could be carried out through components that affect cellular signalling and gene expression.

1.3 Phytochemicals

As “phyto” is the Greek word for plant, these chemicals are accordingly plant specific. Phytochemicals are secondary metabolites of plants, with the purpose of protecting the plant from different types of stress, for instance radiation, microorganisms and insects. Several phytochemicals possess bioactive properties that have been associated with reduced risk of many diseases (3-5).

The first documented discovery of phytochemicals was made by the Hungarian biochemist Dr. Szent-Gyorgyi in 1936. He found that compounds isolated from citrus fruits very efficiently improved capillary fragility, and he identified the active substances to be pure flavons or flavonol glycosides (6). He suggested that these compounds were of vitamin nature, and named them “vitamin P” for “permeability”. Phytochemicals have been subject to extensive research during the last decades, and thousands of compounds have been isolated from plant foods. However, more than seventy years after Dr. Szent-Gyorgyi’s discovery, previously unidentified phytochemicals are still being isolated and their bioactive properties investigated.

Figure 1.2 shows one classification of dietary phytochemicals (7). The phenolics and the carotenoids are the most studied groups.

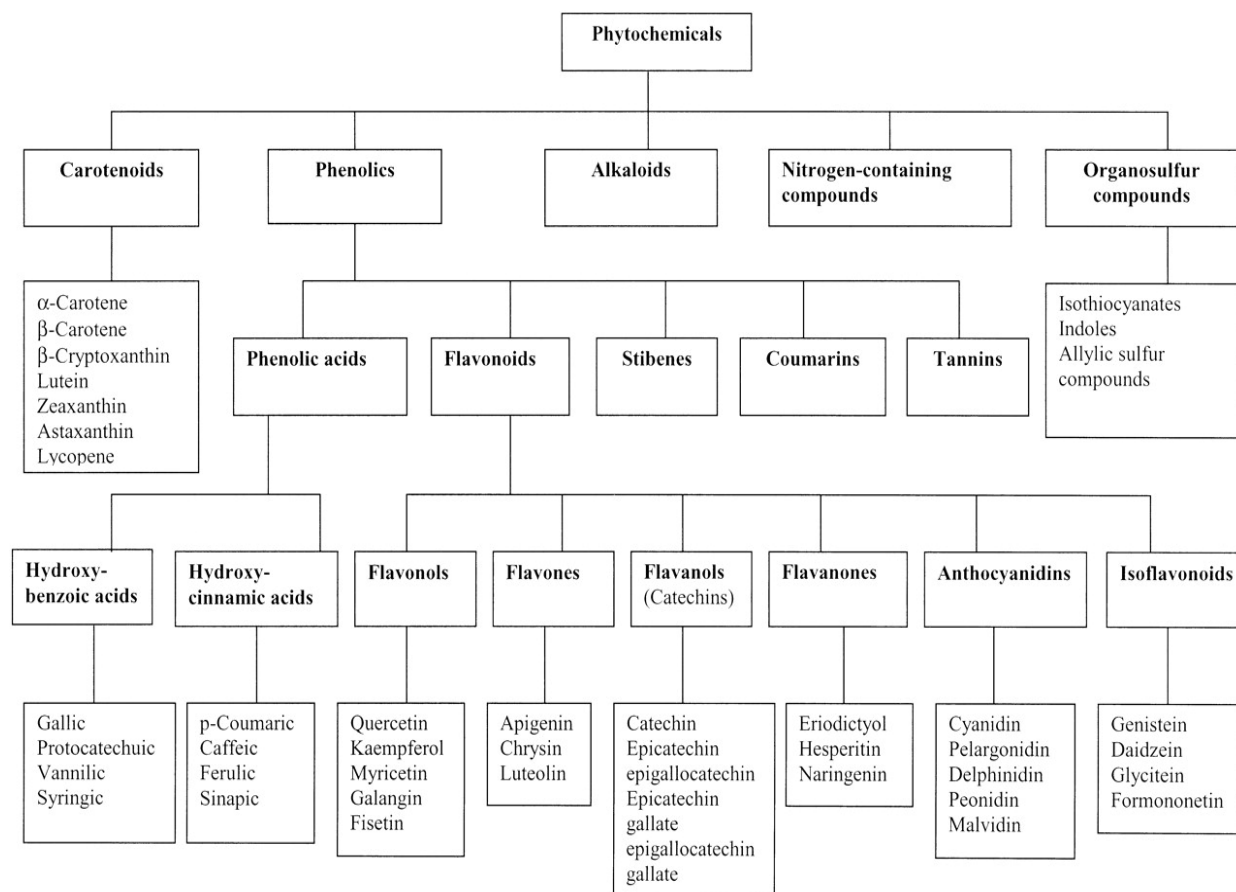


Figure 1.2. Classification of dietary phytochemicals. From Liu (7).

Alkaloids, organosulfur compounds and other nitrogen-containing compounds

Secondary plant metabolites containing nitrogen make a large and extremely diverse group of phytochemicals. They exhibit many different functions in plants, such as defence against herbivores and microorganisms, chemical attractants in flowers and mechanisms for transport and storage of nitrogen (8). The main groups are alkaloids, amines, non-protein amino acids, cyanogenic glycosides and glucosinolates (8). Only a few of these compounds have been extensively studied with respect to biological activity.

The alkaloid caffeine has been extensively studied because of its widespread consumption through coffee and tea, in particular. Caffeine consumption has been studied in association with diseases such as coronary heart disease, arrhythmia and pancreatic cancer, but studies have failed to show definitive correlations (9).

Animal studies suggest that caffeine can provide protection against skin cancer both by ingestion and topical application (10). A human cross-sectional study revealed that consumers of caffeinated coffee have a lower prevalence of skin cancer, and that risk decreases further with increased intake, whereas this effect was not shown for those drinking decaffeinated coffee (10).

Sulphoraphane, an organosulfur compound of the glucosinolates present in broccoli, has been shown to protect against tumorigenesis in animal models, and epidemiological studies have also shown that a diet rich in broccoli can reduce cancer risk (11).

Carotenoids

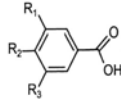
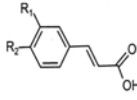
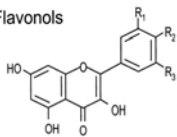
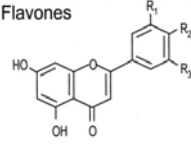
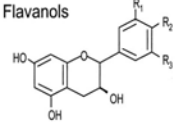
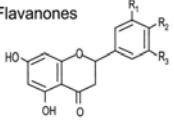
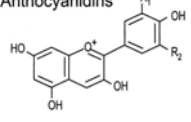
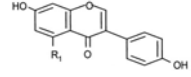
Carotenoids are lipophilic pigments present in plants, microorganisms and animals. They are responsible for many of the red, orange and yellow colours found on fruits and plants, as well as some fish, insects and crustaceans (12). They play important roles in protecting the organism from light damage by scavenging singlet molecular oxygen and peroxy radicals (7;12). Most carotenoids are built up of a 40-carbon skeleton of isoprene units, with a pattern of conjugated double bonds along the backbone, causing different light absorption and antioxidant properties. More than 600 different carotenoids have been identified; α -carotene, β -carotene, β -cryptoxanthin, lutein, zeaxanthin, astaxanthin and lycopene being the most common in our diet (7). Ingested carotenoids can have pro-vitamin A (α -carotene, β -carotene, β -cryptoxanthin) roles. Because of their lipophilic nature and antioxidant capacity, the carotenoids are thought to play an important role in protecting cellular membranes and lipoproteins against oxidative damage (12). One of the most extensively studied carotenoids is lycopene. Several studies have reported that lycopene intake is inversely related to many types of cancers, such as prostate, breast, cervical, ovarian and liver cancer (13).

Phenolics

The most abundant antioxidants in the diet are the phenolics, and several hundred different types have been identified in food materials so far (4). The main types are flavonoids, phenolic acids, stilbenes, coumarins and tannins (7). Flavonoids and

phenolic acids make up the lion's share of phenolics in a regular diet. Table 1.1 presents an overview of common phenolics in foods.

Table 1.1. Overview of some common phenolics and example food sources. (7;14-17).

Polyphenol	General chemical structure	Example food sources
Phenolic acids		
Hydroxy benzoic acids	Hydroxybenzoic acids	
Gallic acid		Tea, spices
Protocatechuic acid		Red berries, onion, spices
Hydroxy cinnamic acids	Hydroxycinnamic acids	
Caffeic acid		Fruits, spices
Chlorogenic acid		Coffee
Ferulic acid		Cereals
Flavanoids		
Flavonols	Flavonols	
Quercetin		Onion, tea, apples
Kaempferol		Tea, spices
Myricetin		Tea, coffee
Flavones	Flavones	
Apigenin		Parsley
Luteolin		Celery
Flavanols	Flavanols	
Catechin		Green tea, chocolate, spices
Epicatechin		Fruit
Flavanones	Flavanones	
Eriodictyol		Lemon
Hesperitin		Orange
Naringenin		Grape fruit
Anthocyanidines	Anthocyanidins	
Cyanidin		Red berries
Peonidin		Wine, cherry
Delphinidin		Wine, blueberry
Isoflavones	Isoflavones	
Daidzein		Soybean
Genistein		Soybean
Glycitein		Soybean

Phenolic acids are divided into hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acid content in foods are generally low, except from onions and red berries (14). Hydroxycinnamic acids are more common in foods. They are present in most fruits and are also found in high levels in cereals (14).

Flavonoids have a common structure where two aromatic rings are linked by three carbon atoms in an oxygenated heterocycle ring. They are classified into six subclasses, flavonols, flavones, flavanols, flavanones, isoflavones and anthocyanins, depending on the structure of the heterocycle ring (7). Flavonols are the most common group of flavonoids in foods (14).

Many phenolics have been extensively studied, but there is a lack of conclusive evidence concerning their role in disease prevention. A protective effect of a compound can be accompanied by an adverse effect, e.g. for resveratrol, that has shown an anticarcinogenic effect, but also increase the risk of cardiovascular disease in mice (4). Clinical studies have often shown contradictory results, and the possible relationship between reduced disease risk and the phenolics mechanisms of action is still unclear (4).

1.4 Phytochemicals and diet

1.4.1 Bioavailability

When dietary plants are eaten, only a fraction of available micronutrients and phytochemicals are absorbed (18). After absorption, there are several processes contributing to the total bioavailability of a compound. A definition of bioavailability is given by the acronym LADME (18):

L = Liberation, the release of a compound from the food matrix
A = Absorption, diffusion or transport from digestive system into circulation
D = Distribution, transport from circulation into tissue
M = Metabolism, conversion and breakdown of the molecule
E = Excretion, the elimination of the compound or its metabolites from the body

Figure 1.3. The linked processes that together define bioavailability. From Holst et. al (18).

Up to several grams of phytochemicals may be ingested every day through eating a regular diet, however the concentrations measured at systemic and tissue levels are in low micromolar ranges (14). There are many reasons for this. First of all, to be absorbed the phytochemical needs to be accessible. If the compound is trapped within the matrix of the food, it will pass through the digestive tract unabsorbed. Accessibility can be greatly influenced by food processing and digestion in the gut. One example is the carotenoid lycopene in tomatoes, which is available in a much larger amount after heat treatment, as this releases the compound from the tomato peel (19). The mechanisms involving intestinal absorption of phytochemicals are still not fully understood. Most of the phenolics are hydrophilic molecules which cannot pass the intestinal wall by diffusion, and specific membrane carriers to facilitate transport have not been found (14).

Many phenolics are handled by the body as xenobiotics, and undergo phase I and II metabolism in the small intestine and the liver, which results in methylated, sulfated and glucoronidated versions of the compounds. This process reduces the possible toxic potential and results in stable, water soluble metabolites (14). Phenolic metabolites in circulation are mainly bound to albumin. The effect of the binding is not clear, but could possibly facilitate antioxidant effects to the interface between lipophilic and hydrophilic phases (14). To what extent phenolics are absorbed in tissue is still not thoroughly investigated. However, accumulation of phenolic metabolites in tissue has been reported, suggesting that they might be able to exert tissue specific effects (14).

Carotenoids do not undergo conjugation during metabolism. They are incorporated unchanged into micelles of dietary fat and bile acids, which are absorbed by passive diffusion into the mucosal cells. The micelles are taken up in the chylomicrons and released into the lymphatic system. In the liver, they are incorporated into the lipoproteins, and released into circulation. The adipose tissue is the main storage site of carotenoids (13).

Metabolites of phytochemicals can be excreted from the body by biliary and urinary elimination. Small conjugates are normally excreted in urine, whereas larger, more

complex metabolites are preferably removed by bile (14). The compounds that are not absorbed may still play a significant role by local effects in the gut. As the gut has an important role in the immune defence, this may lead to indirect systemic influence (18). In the colon, microorganisms break down the phytochemicals and form new, possibly bioactive products. The microbial metabolites are absorbed and conjugated, and the products from colonic microflora can have specific activities. One example is the microbial transformation of soy isoflavones to equol, a nonsteroidal estrogen compound probably responsible for the pseudo-hormonal effect of soy (18).

1.5 Oxidative stress and antioxidant defense

1.5.1 Oxidative stress

Oxidant agents include reactive oxygen species (ROS) and reactive nitrogen species (RNS), and are recognized to have both beneficial and detrimental roles in living organisms. Oxidant agents are radicals, molecules that contain unpaired electrons which makes them highly reactive. The oxidant agents originate from endogenous and exogenous sources. Mitochondrial respiration generates ROS, the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) from molecular oxygen (O_2) in the electron transport chain. Mitochondrial nitric oxide synthase activity generates RNS. Other cellular processes such as metabolism of xenobiotics, inflammatory reactions and peroxisomal β -oxidation of fatty acids also generate ROS (20). Exogenous inducers of ROS can be environmental pollutants, smoking, xenobiotics, UV radiation, alcohol and infectious agents (21-23). When there is an overproduction of ROS, and the presence of antioxidants is too low, oxidative stress can occur. Oxidative stress can damage or inhibit the normal function of important cell components such as lipids, proteins and DNA. On the other hand, production of ROS is essential in e.g. defence against microbes or function as second messengers in cellular signalling (23). There is a delicate balance between harmful and essential levels of ROS, and living species utilizing oxygen have evolved endogenous systems to regulate the amount of free radicals in the cells.

1.5.2 Antioxidant defense

Both non-enzymatic antioxidants and enzymatic antioxidants can contribute to the elimination of ROS or to the prevention of ROS production.

Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)(24). SOD is a very effective endogenous antioxidant, catalyzing the reaction where O_2^- is converted to the less reactive H_2O_2 and O_2 .

There are three types of SOD in humans, cytosolic, mitochondrial and extracellular, and aberrant levels of mitochondrial SOD are correlated with certain types of cancer (25). Catalase is located in the peroxisomes of the cell, where it catalyzes the conversion of H_2O_2 to water and molecular oxygen. Some tumours may have a reduced ability to convert H_2O_2 , caused by a reduced level of catalase (25).

Glutathione peroxidase is a family of both intra -and extracellular enzymes, reducing both hydrogen peroxide and lipid hydroperoxides (26).

Non-enzymatic antioxidants are supplied both from the diet and from endogenous production. There is extensive interaction and co-operation between them, and they are likely to co-operate with the antioxidant enzymes in the management of oxidative stress (26). Vitamin C, vitamin E, glutathione, thioredoxin and lipoic acid are assumed to be some of the important non-enzymatic antioxidants (26).

1.5.3 Antioxidants in disease prevention

Many diseases include oxidative damage as an initial event or in early stages of disease development. “The antioxidant theory” ascribed natural processes like senescence and development of degenerative diseases to cellular oxidative stress. The *in vitro* antioxidant properties of many phytochemicals and some vitamins brought about the use of “antioxidants” as a collective term (18). The protective effects of dietary plants have partly been attributed the antioxidants hypothesized ability to reduce reactive oxygen species *in vivo*, thereby preventing oxidative stress.

Dietary antioxidants have been proposed as a potential “magic bullet”, possibly responsible for the protective properties of phytochemical rich foods towards several degenerative diseases. Antioxidant intervention has been a major focus in dietary disease prevention, and the commercial industry producing antioxidant supplements has had a period of tremendous growth.

Early hypotheses of the phytochemicals protective effect have been heavily challenged. *In vitro* and animal studies have reported protective effects that have not been reproduced in humans, making it difficult to evaluate the phytochemicals role in disease prevention (4). Several intervention studies have failed to show protective effects of antioxidant supplements *in vivo* (27-29). These studies were designed to show protective effect of high doses of antioxidant phytochemicals and vitamins, alone or in combination. Meta analysis of antioxidants supplement trials suggest that such high dose-supplements might even be harmful (27-29). Several micronutrients follow a u-shaped curve, ranging from risk of deficiency in one end to possible adverse effects in the other. The “window” of optimal, protective level can vary a lot between compounds, and for many phytochemicals these ranges have not yet been assessed (18).

Several problems need to be addressed before conclusions can be drawn, concerning how the antioxidant properties of phytochemicals may contribute to the protective effect of dietary plants:

- Whether small molecule antioxidants, which work as antioxidants *in vitro*, also have the same effect *in vivo*, is not established (18;30).
- Some phytochemicals undergo extensive metabolism during absorption, making the molecular effects found *in vitro* potentially irrelevant *in vivo*.
- The possible combinatorial and/or synergistic effects between metabolites are very complicated to measure (30).

- The phytochemicals are present in blood and tissue at concentrations far below those of endogenous antioxidants such as glutathione. In this context, the contribution of dietary antioxidants to the total antioxidant potential might be next to negligible (18).
- The effects of dietary antioxidants may be related to interactions with intracellular signal cascades (18).

1.5.4 Gene regulation

Cells communicate and respond to extracellular stimuli by signal transduction. This process enables the cell to transmit information from the outside to functional elements inside the cell. Extracellular signalling molecules such as cytokines, hormones, growth factors and neurotransmitters activate cell signalling pathways, leading to activation of target gene transcription (23).

Many phytochemicals with *in vitro* antioxidant properties have functions beyond radical scavenging. More and more studies suggest that the protective effects of phytochemicals can be related to the ability to modulate signal transduction pathways (31). Phytochemicals or their metabolites may influence transcription of genes by activation or repression of transcription factors, effects that can occur at concentrations much lower than what is relevant for antioxidant activity (30). As dietary plants contain an abundance of phytochemicals, the combination provided by a varied diet can affect the genetic machinery by a multitude of possible mechanisms of action.

Phytochemicals processed by the body via xenobiotic pathways may activate specific or general cellular response to oxidative stress and toxin exposure. In this way, the phytochemicals could act as indirect antioxidants. Thus the phytochemicals may prepare the cell for more severe oxidative stress by activating signalling pathways that lead to upregulation of cytoprotective proteins. This phenomenon has been described as “preconditioning” or “hormesis” (32).

The term hormesis has been used to describe the adaptive response of cells and organisms to moderate stress, and can explain the biphasic dose response curve of chemicals that have stimulatory effects at low doses, but are toxic at high doses (32). Hormetic pathways activated by phytochemicals can induce expression of genes that code for antioxidant and cytoprotective enzymes (32). An example of this is the activation of the Nrf2/Keap1 (nuclear factor erythroid2-related factor/kelch like ECH-associated protein 1), pathway, which can be activated by e.g. sulforaphane or curcumin, phytochemicals present in broccoli and turmeric, respectively (18;32). Nrf2 regulates expression of a large number of genes in antioxidant function and xenobiotic detoxification. Also, the nuclear factor kappa B (NF- κ B), an important transcription factor regulating cellular stress-, immune- and inflammatory responses, can be modulated efficiently by phytochemicals and phytochemical-rich plant extracts (33;34).

1.6 The NF- κ B family of transcription factors

NF- κ B constitutes a family of transcription factors that are expressed in most cell types and involved in a large number of cellular processes such as inflammatory responses, anti-apoptosis, cellular growth and differentiation (35). The wide range of inducers and the many target genes it affects bring about an understanding that NF- κ B is a general mediator of stress response in the organism.

Activation of NF- κ B plays an important role in defence against bacterial or viral infections, and in regulating innate and adaptive immune responses in states of inflammation (36). Increased NF- κ B activity in inflammatory conditions is a transient and self-limiting event, and reflects a normal immune response in the organism. However, aberrant activation of NF- κ B is linked to many diseases with an inflammatory element, such as rheumatoid arthritis, asthma, atherosclerosis, Alzheimers disease, diabetes and various types of cancer (37;38). These discoveries have triggered extensive research into the transcription factor NF- κ B, and great effort

has been put into elucidating the molecular details of the signalling pathways and their regulation.

1.6.1 The NF- κ B proteins and signalling pathways

In mammals, the NF- κ B family consists of five members: Rel, RelA, RelB, p50 and p52 (39). They share a Rel homology domain (RHD), which is highly conserved in mammalian evolution. The RHD inhabits many domains that provide functionality, such as DNA binding, dimerisation, interaction with inhibitory proteins and nuclear localisation signal (NLS) (38). The NF- κ B proteins form homo–and heterodimeric transcription factors. Most combinations can occur, with the exception of RelB which dimerises only with p50 or p52. The most common dimer is p50/RelA, and the term NF- κ B is often used synonymously with p50/RelA. Dimer formation is necessary for DNA binding, as each subunit binds to one half site of the κ B binding site (38).

The activity of NF- κ B is regulated in a way that allows rapid cellular response to stimuli that are perceived as threats to the organism. In non-stimulated cells, the NF- κ B resides in the cytoplasm, bound to inhibitors of NF- κ B, the I κ B proteins. The NF- κ B/I κ B complex exist as a trimer (one NF- κ B dimer bound to one I κ B), or a heterodimer (one NF- κ B monomer bound to an unprocessed precursor). The I κ B proteins inhibit activation of NF- κ B by impeding DNA binding and masking the NLS in the RHD (38). There are at least seven mammalian members of the I κ B proteins, I κ B- α , I κ B- β , I κ B- γ , I κ B- ϵ , Bcl-3, p105 (precursor of p50), p110 (precursor of p52) and I κ BR (38), which have different affinities for individual NF- κ B protein combinations (33). Common features are the ankyrin repeats necessary for RHD interaction, the C-terminal acidic region necessary for inhibition of DNA binding and the C-terminal PEST-sequence (proline (P), glutamate (E), serine (S), and threonine (T) rich sequence) involved in protein degradation (38). The NF- κ B and the I κ B proteins are shown in figure 1.4 (39).

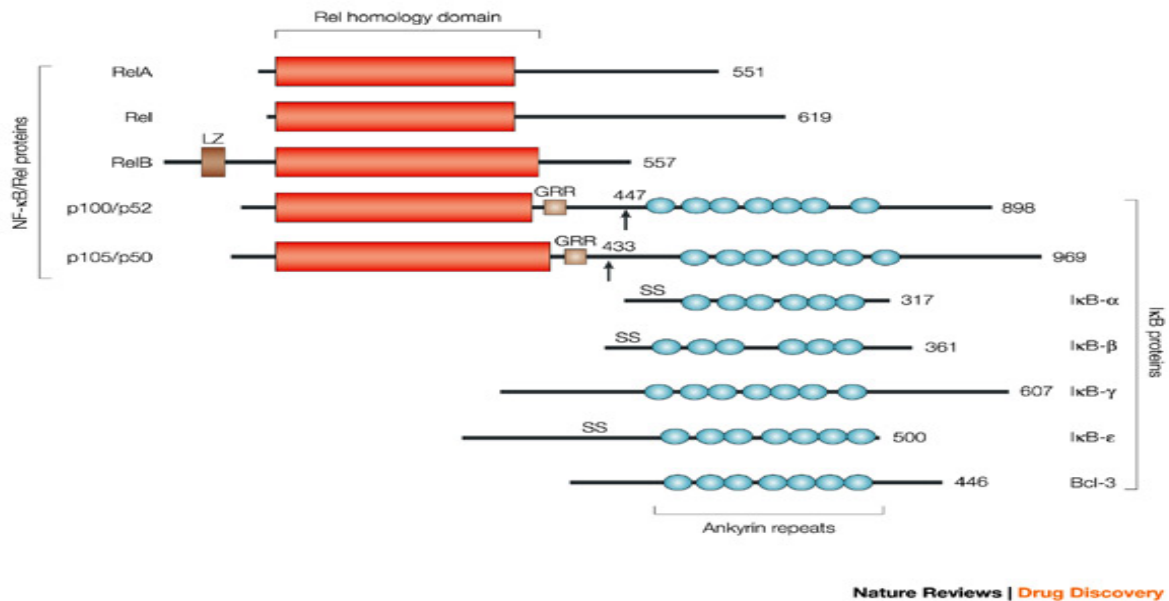
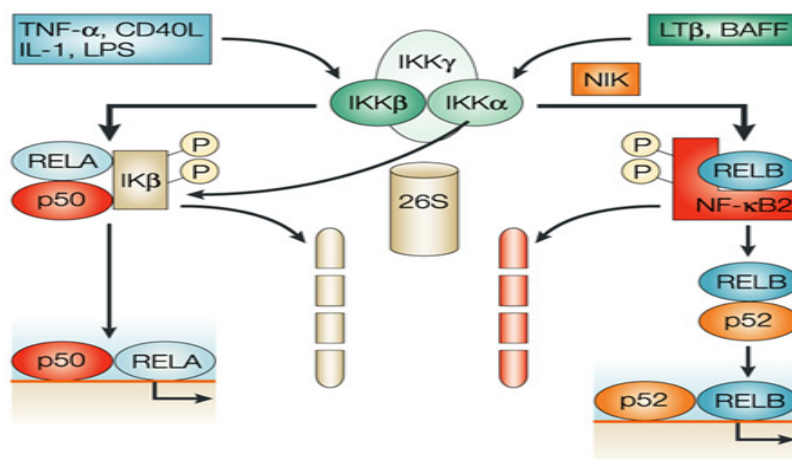


Figure 1.4. Schematic structure of the NF-κB and the IκB proteins. From Karin et al. (39).

NF-κB can be activated by a large variety of stimuli, such as inflammatory cytokines, microorganisms, viruses, heavy metals, cigarette smoke, environmental toxins, xenobiotics and radiation (40;41). Through membrane bound receptors, extracellular signals lead to activation of an IκB kinase complex (IKK), which catalyses the degradation of the IκB protein. The IKK complex generally contains two kinase subunits, IKKα, IKKβ and a regulatory subunit called NEMO (NF-κB essential modulator). The kinase subunits of the IKK complex phosphorylate serine residues on IκB, which in turn lead to ubiquitination and eventually degradation by proteasomes. Liberated NF-κB dimers can then enter the nucleus and bind κB sites on the DNA. There are two main pathways for NF-κB signal transduction; the classical and the alternative (42), illustrated in figure 1.5.



Nature Reviews | Cancer

Figure 1.5. Two main pathways lead to activation of NF-κB. The classical pathway (left), and the alternative pathway (right) From Karin et. al (35).

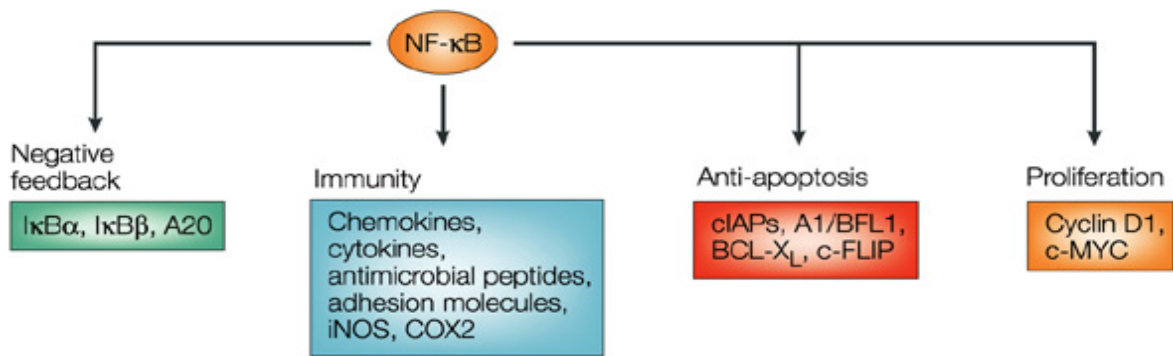
The classical pathway is triggered by stimuli such as tumour-necrosis factor- α (TNF α), CD40 ligand (CD40L), interleukin-1 (IL-1) and lipopolysaccharide (LPS) (35), which leads to recruitment of several signal transducing adaptor proteins, before the IKK complex phosphorylates the I κ B protein (42).

The alternative pathway is independent of the NEMO regulatory subunit, leads to phosphorylation of an IKK α dimer, and is triggered by members of the tumor necrosis factors (TNF) family other than TNF α . These factors specifically activate the IKK α (35). The alternative pathway is also dependent on adaptor proteins, as well as a NF- κ B inducing kinase (NIK) (42).

Also, a third pathway has been described, “the atypical pathway”, which is independent of the IKK and triggered by DNA damage, e.g. from UV radiation (42).

1.6.2 The NF-κB target genes

Activated NF-κB migrates to the nucleus and can activate the expression of more than 150 genes (43) that are divided into four classes according to functionality: negative feedback, immunity, anti-apoptosis and proliferation (35). Figure 1.6 gives a brief overview of some of the target genes.



Nature Reviews | Cancer

Figure 1.6. The four major groups of NF-κB target genes. From Karin et. al (35).

The negative feedback genes ensure that the response is attenuated, as IκB synthesis is a result of NF-κB activation. Newly synthesized IκB will inactivate remaining NF-κB in the cytoplasm, and also bind and inactivate DNA bound dimers in the nucleus (38). Concerning the immune response, NF-κB is both being activated by inflammatory cytokines, as well as inducing the expression of several cytokines and chemokines. Thereby the signal can spread from cell to cell. The immune response produces cytokines, chemokines, adhesion molecules, acute-phase proteins and pro-inflammatory enzymes. The anti-apoptosis and proliferation groups of target genes are important for cell survival under stress, to ensure that the cell does not unnecessarily succumb to the stress (43).

Pahl et al. (43) have reviewed the NF-κB target genes that are activated upon NF-κB signalling in a given cell. The specificity of the response is maintained by receptors and signal transduction molecules present in each cell type. There can be regulation at the transcriptional level, as some promoter/enhancer regions may require combined binding of additional transcription factors to trigger transcription of the target gene. If the additional contributors are not present, the NF-κB signal might not have an effect. The combinations of NF-κB dimers can also have preferences for binding sites on DNA, adding another level of selectivity. Possibly, the influence of NF-κB activation is even greater, as some of the target genes are transcription factors themselves, making NF-κB also an indirect inducer of transcription. Examples of this are the proto-oncogene *c-myc* and the tumor suppressor gene p53.

1.6.3 NF- κ B and cancer

Cancer is not one, but many diseases, each with different characteristics and treatment options. The development of cancer can be influenced by a vast amount of factors. Concerning alterations in cell physiology, there are six hallmarks that collectively inflict malignant growth (44):

- Self sufficiency in growth signals
- Insensitivity to anti-growth signals
- Tissue invasion and metastasis
- Limitless replicative potential
- Sustained angiogenesis
- Evading apoptosis

All six hallmarks can be affected by NF- κ B target genes through the transcription of genes involved in cell proliferation, angiogenesis, metastasis, inflammation and suppression of apoptosis (31), making this transcription factor a promising target in chemoprevention.

Aberrant activation of NF- κ B can arise from different defects in the signalling system. One example is genetic abnormalities, such as mutated or truncated I κ B, which causes Hodgkin's lymphoma, a cancer of the lymph nodes. Also, overexpression of NF- κ B or constitutive I κ B kinase activity can contribute to cancer development (45).

The pharmaceutical industry has made major efforts in developing efficient inhibitors of NF- κ B. These include IKK inhibitors, NF- κ B RNAi (RNA interference; degradation of NF- κ B mRNA) and proteasome inhibitors (39). In 2006, Gilmore and Herschovitch (33) listed in 785 known inhibitors of NF- κ B, and this number has probably increased since. Inhibitors include both natural and synthetic compounds, targeting the signal transduction at different steps.

1.6.4 Modulation of NF- κ B by diet

Among the natural NF- κ B inhibitors are many compounds originating from dietary components, and several dietary plants are efficient inhibitors of NF- κ B activation *in vitro*. Table 1.2 presents some examples of dietary NF- κ B inhibiting compounds.

Table 1.2. Dietary components that have shown inhibitory effect on NF- κ B (34;37;46).

Compound	Origin
Anethole	Fennel, anise, clove
α -Tocopherol	Nuts, grains, oils
Apigenin	Oregano, onion
Caffeic acid	Coffee, fruits, oregano, thyme, walnuts
Capsaisin	Chilli
Carnosol	Rosemary, thyme
Clorogenic acid	Coffee, tea, walnuts
Curcumin	Turmeric
Eugenol	Clove, sweet basil
Epigallocatechin	Green tea
Genistein	Soy
S-allyl-cysteine	Garlic
Sulphoraphane	Cruciferous vegetables
Quercetin	Onions, clove

Paur et al. showed that extracts of oregano, thyme, clove, turmeric and coffee inhibited NF- κ B activation in a monocytic cell line (34). The NF- κ B modulating effect of these plant extracts may be mediated by the contents of known inhibitory compounds, as they all contain one or more of the compounds listed in table 1.2. Paur et al. also reported ten additional plant extracts with the dual ability to induce a basal activation, and inhibit NF- κ B activation upon stimulation with bacterial lipopolysaccharide (LPS). Allspice, crowberry, walnut, wild strawberry and sunflower seeds were the most potent inducers of basal NF- κ B activity (34).

The majority of experiments concerning modulation of NF- κ B have been performed by testing isolated dietary compounds *in vitro*. Such a reductionistic approach can

cause additive, synergistic or antagonistic effects from whole foods to be disregarded. Foods with NF- κ B modulating properties could have potential in prevention of disease, in delaying the disease onset or as therapeutic agents (37). Results from *in vitro* models need to be confirmed in animal and human studies before conclusions concerning the efficiency of NF- κ B modulators can be drawn.

2. Aim of the thesis

This thesis adds to a series of projects that study the protective effects of dietary plants on human health. The search for dietary factors that inhibit or modulate NF- κ B activation can be useful both in prevention and treatment of diseases. Earlier studies conducted by the Rune Blomhoff research group have shown that dietary plant extracts and phytochemicals hold NF- κ B modulating properties in a monocytic cell line.

This Master project was set up to continue the previous work by testing the effects of the best *in vitro* NF- κ B modulators *in vivo* in transgenic NF- κ B-luciferase reporter mice.

The aim of this master thesis is to:

- Test the effect of a combination extract, compiled of the most potent *in vitro* NF- κ B inhibitors, in transgenic reporter mice.
- Test the NF- κ B modulating ability of three of the constituents from the combination extract one by one in transgenic reporter mice.

3. Materials

○ Transgenic mice

Transgenic mice	Distributor	Location
NF- κ B-luciferase mice	Cgene	Oslo, Norway

○ Transgenic mice equipment

Equipment	Manufacturer	Location
Cameo 25 Gas Syringe Filter, 0.22 micron	Osmonics Inc, DGA02025SO	Minnetonka, MN
Centrifuge tubes, 15 and 50 mL	BD Biosciences	Franklin Lakes, NJ
Feeding tube for oral gavage feeding (0.8 – 1.2 mm)	AgnTho's AB	Lidingö, Sweden
Micro tubes 1.5 mL	Sarstedt	Nümbrecht, Germany
Omnifix (syringes) 1 and 10 mL	Braun Melsungen AG	Melsungen, Germany
Sterican (needles) 0.15x16 mm	Braun Melsungen AG	Melsungen, Germany
96 well plates	Sigma, M2936	St.Louis, MO

○ Chemicals

Chemical/compound	Manufacturer	Location
Albumin	Bio-Rad Laboratories Inc., 500-0007	Hercules, CA
Argon	AGA, 100325	Oslo, Norway
ATP (adenine triphosphate)	Roche Diagnostics	Ottweiler, Germany
Coenzyme A	Roche Diagnostics	Ottweiler, Germany
Corn oil Biochemika	Sigma, C-8267	St. Louis, MO
D-Luciferin	Biosynth AG	Staad, Switzerland
DTT (dithiothreitol)	Sigma	St. Louis, MO
EDTA (diethylenediamine tetra-acetic acid)	Merck	Darmstadt, Germany

Ethanol	Arcus AS	Oslo, Norway
Isoflourane	Baxter AS	Oslo, Norway
Methanol (HPLC-grade)	Merck	Darmstadt, Germany
MgSO ₄ • 7H ₂ O	Merck	Darmstadt, Germany
ddH ₂ O	Millipore	Bedford, MA
PBS (Phosphate buffered saline)	Bio Whittaker, BE17-512F	Verviers, Belgium
Reporter Lysis Buffer	Promega, E3971	Madison, WI
Sodiumacetate trihydrate	Riedel-deHaën AG	Seelze, Germany
Tricine	Sigma, T5816	St. Louis, MO

○ Dietary plant extracts

Name	Botanical name	Producer	Location
Combo extract			
Clove	<i>Syzygium aromaticum</i>	Krydd Huset	Ljung, Sweden
Arabica coffee	<i>Coffea arabica</i>	CIRAD	Montepellier, France
Oregano	<i>Oreganum vulgare</i>	Santa Maria	Mölndal, Sweden
Thyme	<i>Thymus vulgaris</i>	Hindu	Bergen, Norway
Walnut	<i>Juglands regia</i>	Diamond	California, USA
Single extracts			
Coffee	<i>Coffea arabica</i>	Friele	Bergen, Norway
Clove	<i>Syzygium aromaticum</i>	Santa Maria, Hindu	Mölndal, Sweden Bergen, Norway
Thyme	<i>Thymus vulgaris</i>	Santa Maria	Mölndal, Sweden
Walnut	<i>Juglands regia</i>	Unknown	California, USA

○ Kits

Kit name	Manufacturer	Location
Bio-Rad Protein Assay	Bio-Rad laboratories Inc.,500-0006	Hercules, CA

○ Instruments

Instrument	Manufacturer	Location
Biofuge Fresco	Heraeus Instruments	Osterode, Germany
2510 Branson	Branson Ultrasonics Corp.	Danbury, CT
IVIS Imaging System 100 Series	Xenogen Corporation	Alameda, CA
Labofuge 400e	Heraeus Instruments	Osterode, Germany
Luminometer TD 20/20	Turner Designs	Sunnyvale, CA
Megafuge 1.0 R	Heraeus Sepatech GmbH	Harz, Germany
Synergy 2	Bio Tek [®] Instruments, Inc	Winooski, VT
Titertek Multiskan Plus	ELFAB	Finland

○ Software

Software	Manufacturer	Location
Living Image Software	Xenogen Corporation	Alameda, CA
Microsoft Office XP	Microsoft Corporation	Redmond, WA
Reference manager 12	ISI Research Soft	Carlsbad, CA
SPSS 16 for Windows	SPSS Inc.	Chicago, IL

4. Methods

4.1 Dietary plants

The choice of dietary plants used in this project was based on inhibitory effects on NF- κ B shown in previous experiments performed in the Rune Blomhoff group.

A screening of plant extracts done by Paur et al. revealed that extracts of oregano, thyme, clove, turmeric, coffee, allspice, crowberry, walnut, wild strawberry and sunflower seeds were able to significantly reduce LPS induced NF- κ B activity *in vitro* (34). A combination extract compiled of some of the best NF- κ B inhibitors, (coffee, thyme, oregano, clove and walnut), showed synergistic effect in *in vitro* experiments. Subsequently, the combination extract was tested in transgenic reporter mice. One of three experimental rounds testing combination extract *in vivo*, amounting to 8 out of 32 animals in total, was performed within this thesis. A logical continuation involved testing the constituents from the combination extract one by one. Extract of coffee had been tested previously in transgenic reporter mice by the Rune Blomhoff research group, resulting in significant reduction in LPS induced NF- κ B activity (47). Single extracts of thyme, clove and walnut were tested in this project.

4.2 Extract preparation

The food materials were bought from local grocery stores in Oslo, from brands sold by most food chains and accessible to most of the Norwegian population. The combination extract was provided by my supervisor Ingvild Paur.

Thyme and walnut were ground with a hand blender to a fine powder. This step assures that as large a portion of the phytochemicals as possible is accessible to the extraction fluid. For clove extracts, ground clove was used.

Equal volumes of double distilled water (ddH₂O) and methanol were used as solvents in the extraction. The dry sample, 10 g, was added 10-20 ml ddH₂O and 10-20 ml

methanol. The amount of liquid varied between the food materials, depending on how much was absorbed by the dry matter. Mixtures were sonicated in 0°C water bath for 30 min, centrifuged for 10 min at 4000 rpm and 4°C before the liquid phase was transferred to small Erlenmeyer flasks. The centrifugation step was repeated once, with 5 min duration. The methanol and most of the water were evaporated on a hot plate under nitrogen gas flow, leaving a viscous fluid in the flasks. The extracts were diluted in corn oil to a total volume of 5 ml, resulting in a final concentration of 2 g of sample per ml extract. The extracts were transferred to airtight tubes, added argon gas, and kept at -70°C.

4.3 FRAP assay analysis

To measure the total antioxidant capacity of the extracts used in the experiments, ferric reducing ability of plasma (FRAP) assay was used. The method was originally developed for use on plasma, but it can be used for other fluids as well. The method is based on a change in absorption that occurs when a ferric tripyridyltriazine (TPTZ- Fe^{3+}) complex is reduced to ferrous tripyridyltriazine (TPTZ- Fe^{2+}). This reduction takes place at low pH, and causes an intense blue colour, with an absorption maximum at 593 nm, to appear. The present antioxidants' reducing ability is the limiting factor, as Fe^{3+} is added in excess, and the blue colour is a measure of total reducing ability of the sample of interest. See table 4.1 for details on the FRAP assay solution.

Plant extracts were diluted 1000× in a solution of 10% ddH₂O and 90 % methanol. Measurements of absorption change were detected by a Technicon RA 1000 system at 600 nm.

Table 4.1. Ferric reduction potential of plasma (FRAP) assay solution.

Reagents	Component	Volume in solution
Acetat buffer	300 mM acetate buffer, pH 3.6 + C2H4O2 16 mL/L buffer solution	25 mL
HCl	40 mM	2.4 mL
FeCl3 x 6 H2O	20 mM	2.5 mL
TPTZ solution	250 mg in 5 mL methanol	155 µl

4.4 Luciferase reporter system

Bioluminescence occurs when luciferases, enzymes capable of producing light, react with specific substances and create a product molecule in an electronically excited state sufficient to emit a photon. The emitted light can be used as a detection system for luciferase activity, and when its genetic expression is controlled by a particular regulatory element, the luciferase acts as a reporter for this element. We have used the luciferase reporter system based on firefly luciferase for both *in vitro* and *in vivo* experiments. The luciferase is encoded by the LUC gene, which is coupled to a promoter with three binding sites for NF-κB. Activation by binding of NF-κB to these sites will result in production of luciferase. When the substrate D-luciferin is added, luciferase catalyzes the formation of oxyluciferin and CO₂, creating a burst of light that decays to a stable, low level of sustained luminescence (48). Chemical reaction:

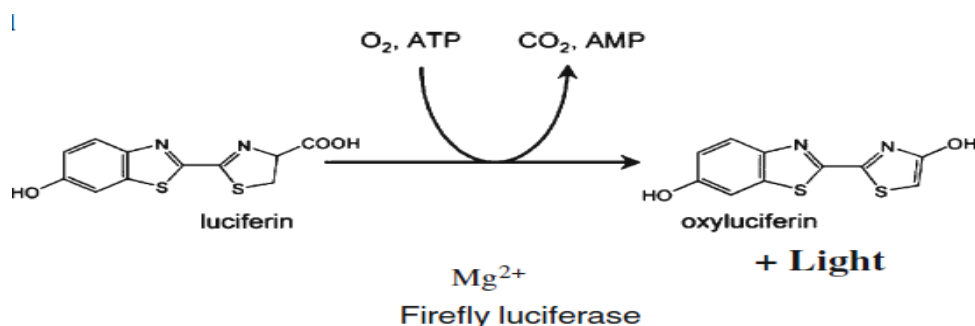


Figure 4.1. Chemical reaction catalyzed by luciferase. From Berger et.al. (49)

The oxidation reaction yields light of 550-570 nm wavelength, and this luminescence can be detected by a sensitive camera (molecular imaging) or by biochemical assays. An advantage of using LUC as a reporter gene is that the bioluminescence has little background, which makes this gene a reliable reporter of activity (50).

4.5 *In vivo* experiments

4.5.1 Mice as a models for human biology

The biology of living organisms is extremely complex, and the metabolic pathways involved in homeostasis interact in seemingly indefinite ways. To elucidate some of the mechanisms present in biological systems, approaching the problem from a simpler starting point can reduce the immensity. Model organisms have been used for this purpose for more than a century, gradually contributing to our understanding of life sciences.

Generally, model organisms should meet certain requirements; e.g. small size, short generation time, possibilities of genetic engineering and limited ethical considerations. Organisms such as the yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* have been very important to elucidate mechanisms of for instance cell cycle, differentiation and the genetics of embryogenesis, respectively. In this type of research, properties such as a small genome and a limited amount of cells are additional advantages.

Research that aims at understanding human physiology or disease development, however, requires increased complexity of the model organism to resemble the human genetics and physiology. Despite having diverged 75 million years ago (51), *Mus musculus* and *Homo sapiens* have a close genetic relationship, making the mouse a good model for human biology. The predicted gene numbers of mice and man have been oscillating, the numbers being continually adjusted, but the latest estimates are 20000-25000 protein coding genes for both species (52;53). Approximately 99 % of

the human genes have murine homologues (54), and this kinship results in many similar or identical biological pathways.

A very useful feature of the mouse is the possibility to alter the genome, and still get fertile and viable progeny. New genetic material can be added by microinjection of zygotes or by embryonic stem cell technology. Genes can be mutated by homologous recombination, providing the opportunity to study the role of one specific gene, a so called “knock out”. Mice have the ability, either naturally or by genetic engineering, to develop human diseases; diabetes, atherosclerosis and heart disease, cancer, hypertension and osteoporosis, to name a few (54). This enables researchers to create models to investigate the mechanistic events in disease development.

Through the years, a vast amount of information and experimental methods concerning mouse genetics have been built up. These are very valuable means for understanding the basis of both diseases and normal biological processes. Other properties that make the mouse a convenient model is its very small size and short generation time for a mammal. It is also relatively cost-effective (54). However, there are considerable ethical considerations concerning the use of laboratory mice.

The animal experiments in this project were performed at the Department of Comparative Medicine. The department maintain strict access regulations to protect the welfare of the animals, and to ensure that only trained personnel are allowed to perform animal experiments. Prior to the start of the project, curriculum of the “laboratory animal science” course was read, and thorough practise training was completed. Based on these preliminary arrangements, access to the department was given.

The mice used in this project were attended to as required by the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). All animal experiments were performed according to national guidelines for animal welfare.

4.5.2 Transgenic NF- κ B reporter mice

Animal models can confirm *in vitro* data and provide new and supplementary information relevant to the *in vivo* situation (40). To test whether results seen in *in vitro* experiments also take effect *in vivo*, transgenic mice expressing luciferase were used. The luciferase production in these mice is activated by NF- κ B activation, as the LUC gene is coupled to a promoter containing three binding sites for NF- κ B. The protocol for generating transgenic mice was developed by Carlsen et al (50). For a schematic overview of the procedure, see figure 4.2. The genetic construct was incorporated into the mouse genome by microinjection of fertilized zygotes of superovulated females. The zygotes were then transferred to pseudopregnant recipients. The offspring (F_0 generation) were tested by polymerase chain reaction (PCR) for presence of the construct. The F_1 generation was analysed for transgenic expression, and crossed with wild type F_1 mice to produce heterozygous individuals (50).

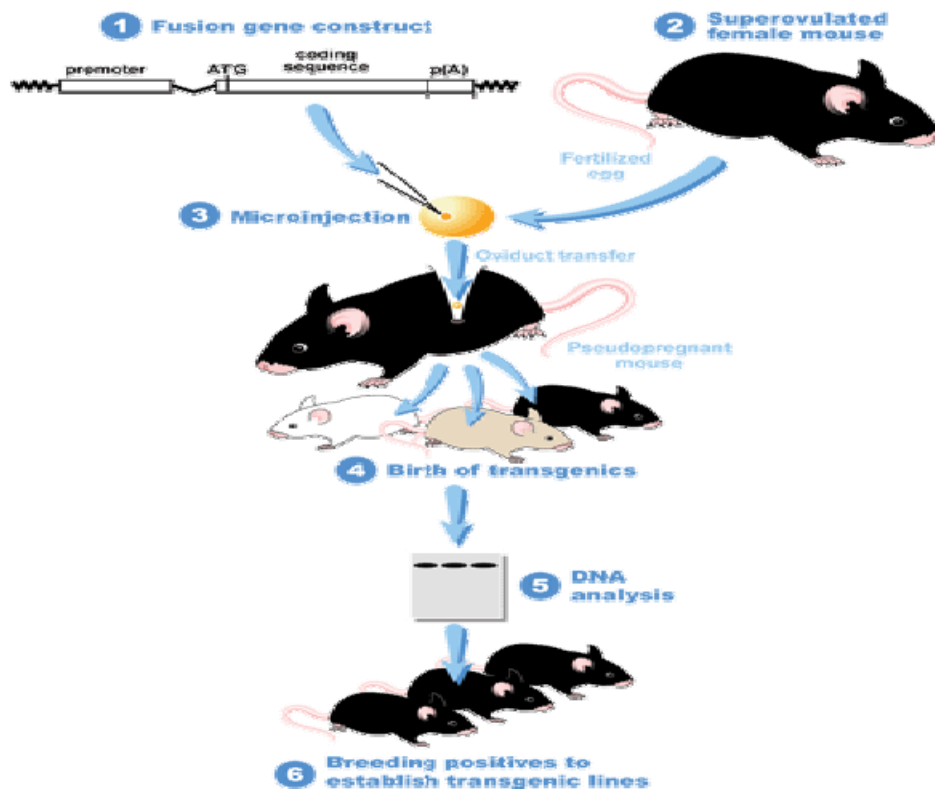


Figure 4.2. Generating transgenic mice. From <http://cancer.ucsd.edu/>. Moores Cancer Centre. San Diego Medical Centre. University of California (55) .

Experiment setup

In each experiment the mice were randomized into a group receiving only corn oil (controls) and a group receiving the relevant extract. The experimental setup is illustrated in figure 4.3.

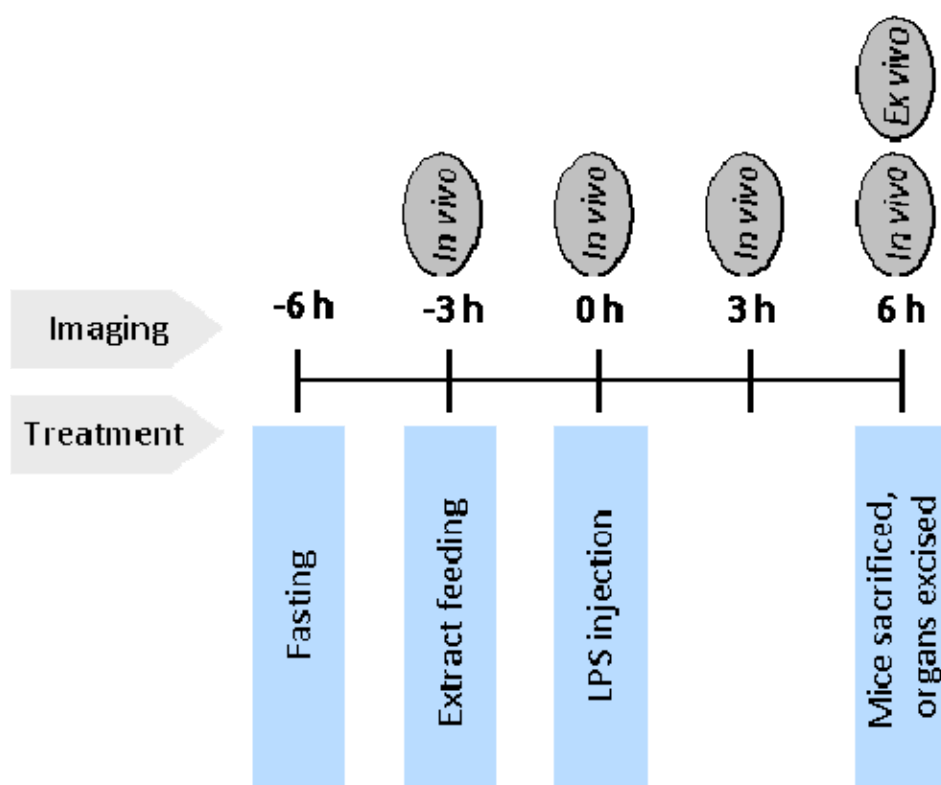


Figure 4.3. The experimental setup of *in vivo* experiments.

To ensure equal absorption conditions, the mice were fasted for 3 h prior to treatment. At time -3 h, the mice were anesthetized with isoflourane and shaved on the abdominal area to make the luminescence visible. Still under anaesthesia, they were injected intraperitoneally (i.p.) with 160 mg/kg D-luciferin. After 7 min incubation following injection the mice were imaged. Then they were fed 300 µl of either extract or control vehicle (corn oil) by oral gavage. See table 4.2 for details on treatments and amounts for experiments.

Table 4.2. Distribution of mice, extracts and amounts used in *in vivo* experiments.

Extract	Contents provided	Dose	Mice
Combination	Thyme, oregano, clove: 60 mg Coffee, walnut: 600 mg	300 µl	16 control, 16 extract (4 control, 4 extract done in this project)
Thyme	Thyme: 600 mg	300 µl	13 control, 12 extract
Clove / Walnut	Walnut: 600 mg Clove: 600 mg	300 µl	9 control, 21 extract (10 clove, 8 walnut)

At time 0 h, LPS (2.5 mg/kg in 100 µl phosphate buffered saline (PBS)) was injected subcutaneously (s.c.) on the back, near the tail.

LPS, a compound found in the outer membrane of gram negative bacteria, is a strong inflammatory inducer involved in the pathogenesis of sepsis. It is recognized by cell receptors and produces a systemic inflammation via two pathways, the TLR4-Bc110 pathway and the ROS-Hsp27 pathway (21). The TLR4-Bc110 pathway involves recognition of LPS components by TLRs (Toll Like Receptors), which trigger the innate immune system (21). Activation of the ROS-Hsp27 pathway might be initiated by ROS-generating interactions with membrane lipids. This pathway is probably leading to decline in Hsp27 (Heat shock protein) phosphorylation and increase in IKK β -IkB α phosphorylation (21). Both pathways are integrated at the IKK signalosome where phosphorylation of units in the IKK complex leads to translocation of NF- κ B to the nucleus.

In vivo imaging was performed at -3, 0, 3 and 6 h as described in figure 4.3. (For the combination extract experiment, *in vivo* imaging was performed at -3, 0, 2, 4 and 6 h, in accordance with the earlier combination extract experiments performed). Injection of D-luciferin and the subsequent incubation was repeated at every imaging time. Directly following *in vivo* imaging at 6 h, the mice were euthanised and the organs (brain, lung, heart, thymus, spleen, kidney, liver, intestines, skin, muscle, adipose

tissue, uterus or testicle and epididymis) excised. *Ex vivo* imaging was done before the organs were frozen in liquid nitrogen (except intestines) and then kept at -70 °C for subsequent luciferase assay and protein content analysis.

4.5.3 Measuring luminescence

In vivo and ex vivo imaging

The IVIS Imaging System was used to detect luminescence in *in vivo* experiments. Molecular imaging allows visualisation of biological processes within an intact living subject, and repeated measurements within the same animal are possible. In this way differences between animals will be less significant, as the animals can serve as their own controls (40).

The imaging system consists of an ultrasensitive camera that detects photons generated by the luciferase activity. Both *in vivo* and *ex vivo* imaging were performed. Emitted light was detected for 1 min for most imaging. A few pictures reached saturation for 1 min exposure, the measuring was then reduced to 30 seconds. Results were processed with Living Image software and presented as intensity maps (visually) or photon counts (numerically).

When measuring *in vivo* luminescence, the mice were given D-luciferin 7 min before they were placed on the back in a light proof chamber. Quantification was done by putting equally sized marking rings on the images (Figure 4.4.). Luminescence emitted from the marked area was measured with Living Image software.

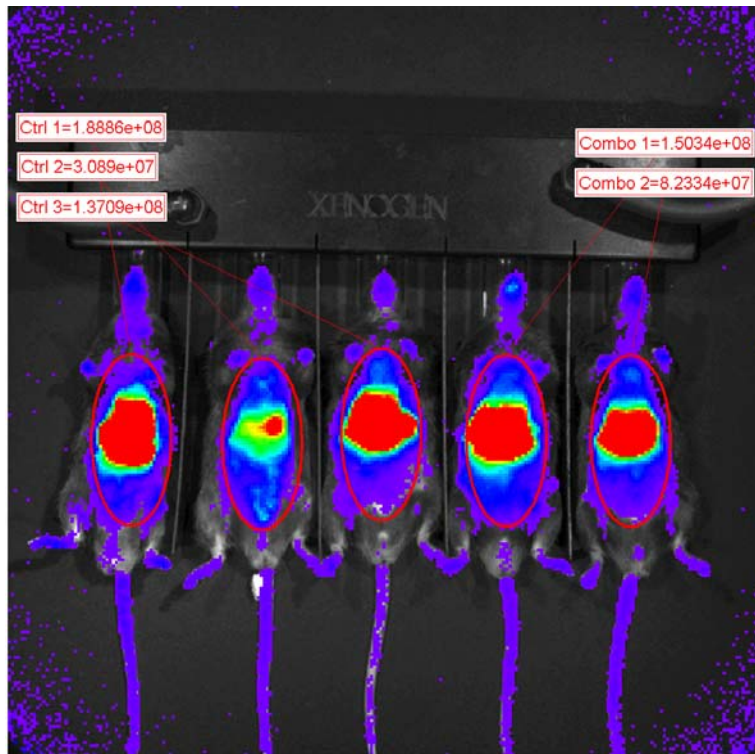


Figure 4.4. Example image of mice marked for *in vivo* measurement in the Living Image software.

Measuring of organs *ex vivo* was performed 30 minutes after injection of D-luciferin. The organs were rinsed in PBS and placed on a black plastic plate in the light proof chamber. Quantification was done by putting marking rings or squares on the images. Grey scale images were used for reference of position. See figure 4.5A. Emitted light was measured with Living Image software. See figure 4.5B.

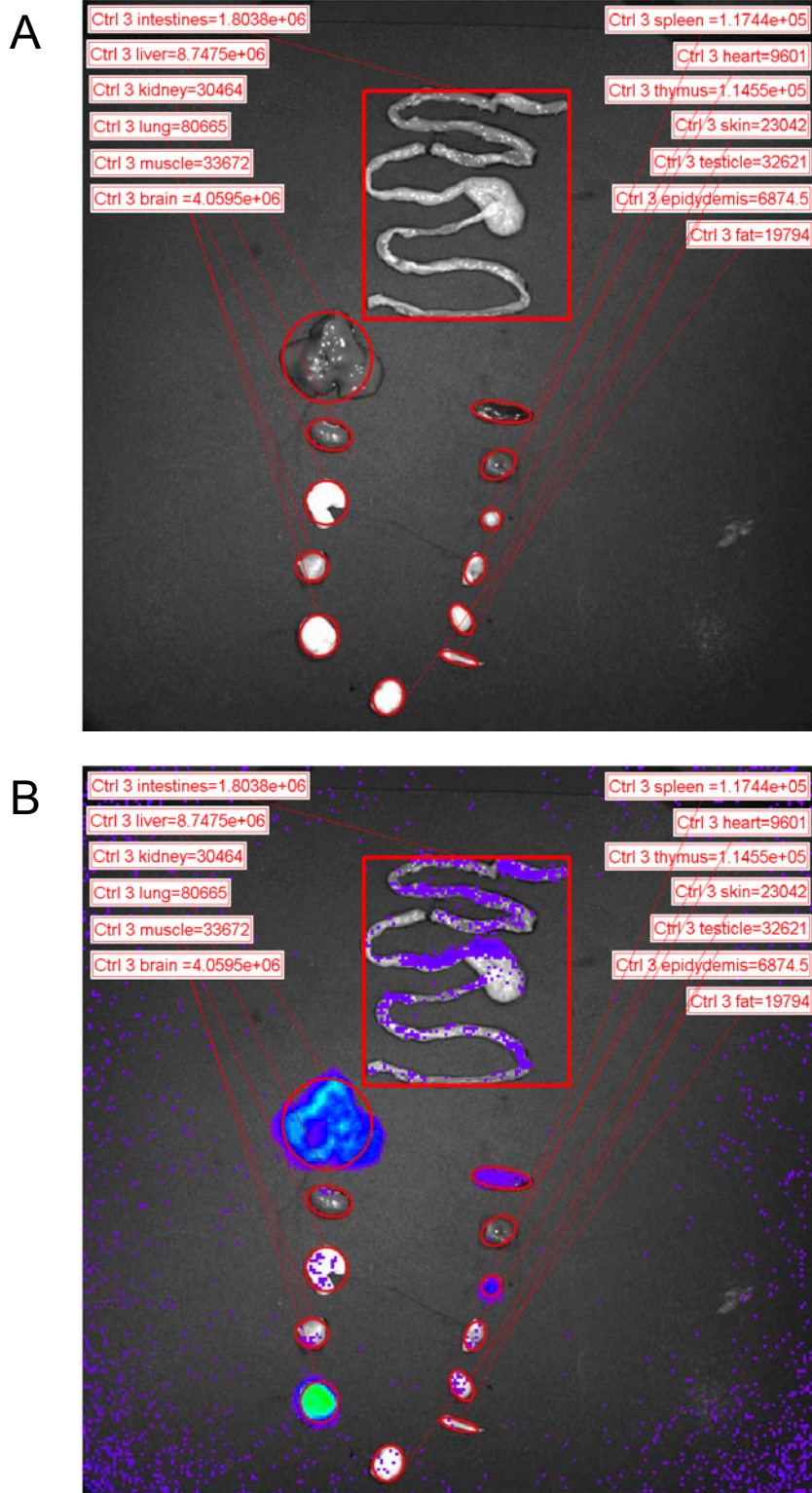


Figure 4.5. Example image of organs marked for *in vivo* measurement in the Living Image software. Grey scale pictures were used for position reference (A). Luminescence measured is visible in the organs (B).

Luciferase assay

Measurements of luciferase activity in organs were also done by a luciferase assay (LUC assay). The organs (brain, lung, heart, thymus, spleen, kidney, liver, intestines, muscle, adipose tissue, uterus or testicle and epididymis) were homogenized in reporter lysis buffer, which breaks down the cell membranes, the homogenates were centrifuged for 15 min at 4 °C, and the supernatant used for measurements. Intestines and skin were excluded from LUC assay due to difficulties in homogenisation, and subsequently unreliable measurements. Luciferase activity was detected in a TD-20/20 luminometer after the addition of D-luciferin-containing assay substrate (Table 4.3). The assay substrate, 100 µl, was added to 20 µl supernatant, in duplicates. Luciferase activity measurements were adjusted according to protein content to even out differences in sample size or homogenisation efficacy.

Table 4.3. Reagents and procedure for making LUC-assay solution.

Reagent	Amount
ATP (Adenosine triphosphate)	52,1 mg
Coenzyme A	20,7 mg
Tricine	358,4 mg
MgSO ₄ x 7 H ₂ O	92,2 mg
DTT (dithiothreitol)	513,5 mg
EDTA (Ethylenediaminetetraacetic acid)	15 µl
ddH ₂ O	80 ml + Adjustment volume
D-luciferin (20 mg/ml)	650 µl
Ingredients were mixed, pH adjusted to 7,4, and ddH ₂ O added to a total volume of 100 ml.	

Protein content

Bio Rad protein assay was used to measure the protein content of the homogenates.

The assay is based on the Bradford assay, a spectroscopic method that detects a shift in absorbance caused by binding of the reagents to basic or aromatic amino acids in proteins. The absorbance, measured at 590 nm, is proportional to the protein content.

The supernatant from the homogenates was diluted 10 to 200 times according to total protein content. Albumin standards to provide a standard curve, and samples, 10 µl of each in triplicates, were put into 96 well plates. Bio Rad assay solution, 200 µl, was added to each well, and absorbance was measured in a Titertek microplate reader using a 590 nm filter.

4.6 Statistical analysis

For *in vivo* experiments, non-parametrical Mann-Whitney U test was performed to test for differences between groups in small experiments with non-normal distributed observations. For comparisons with larger, merged experiments, Student's T-test was used. All statistical analysis was performed with SPSS 16. All analysis were set to $p < 0.05$ significance level.

5. Results

5.1 FRAP analysis

FRAP assay was used to measure total antioxidant capacity of the extracts used for *in vivo* experiments. Results are given as mmol/100g original product, and listed in table 5.1. Clove extract had the highest antioxidant capacity measured, with a FRAP value of 86.9 mmol/100 g. Walnut extract had the lowest FRAP value, 39.3 mmol/100g.

Table 5.1. FRAP values of extracts used for *in vivo* experiments.
(* measurement done in 2008 (56))

<i>In vivo</i> extracts	FRAP (mmol/100 g)	SD (mmol/100 g)
Combination*	82,3	0,9
Thyme	48,5	1,4
Clove	86,9	2,0
Walnut	39,3	10,3

5.2 *In vivo* experiments

The *in vivo* experiments followed as a continuation of experiments previously done on U937 3×κB-LUC cells in the Rune Blomhoff research group. These *in vitro* experiments revealed NF-κB modulating properties of among others thyme, clove, walnut, coffee, and oregano as well as with a combination extract made from all these five. The *in vivo* experiments were designed to test whether the effects on NF-κB activation were reproducible in an animal model. The extracts tested in transgenic reporter mice included the combination extract, and extracts of thyme, clove and walnut one by one.

The mice were given 300 µl extract by gavage feeding in a single dose. Control mice were given 300 µl corn oil. All treatments received LPS, (2 mg/kg), and NF-κB activation was monitored by *in vivo* imaging at 0, 2, 4 and 6 hours for combination extract and 0, 3 and 6 hours for thyme, clove and walnut.

5.2.1 Effect of combination extract on LPS-induced NF- κ B activity

Transgenic NF- κ B-luciferase reporter mice were given combination extract per os in a single dose 3 h prior to LPS injection (0h). NF- κ B activation was measured by *in vivo* imaging at -3, 0, 2, 4, 6h, *ex vivo* imaging (at 6h) and LUC assay of individual organs. Sample size: n=16 control, n=16 extract. Presented here are the combined results of all three repetitions of the experiments, as presented in the submitted manuscript (see appendix 2). The isolated results from the experiment performed within this thesis are attached in appendix 1.

In vivo imaging

Quantification of light emitted from whole mice revealed inhibition of NF- κ B activation in the mice receiving the combination extract compared to control mice.

One representative mouse from each treatment is shown in **figure 5.1A**.

At 2 h ($p=0.043$) and at 6 h ($p=0.041$), the extract significantly inhibited the LPS-induced NF- κ B activation in the extract group compared to the control group by 40 % and 36 %, respectively (**Figure 5.1B**). Based on the area under curve (AUC) for 0-6 h, the reduction in the extract group is 35 % ($p=0.040$) compared to the control group (**Figure 5.1C**).

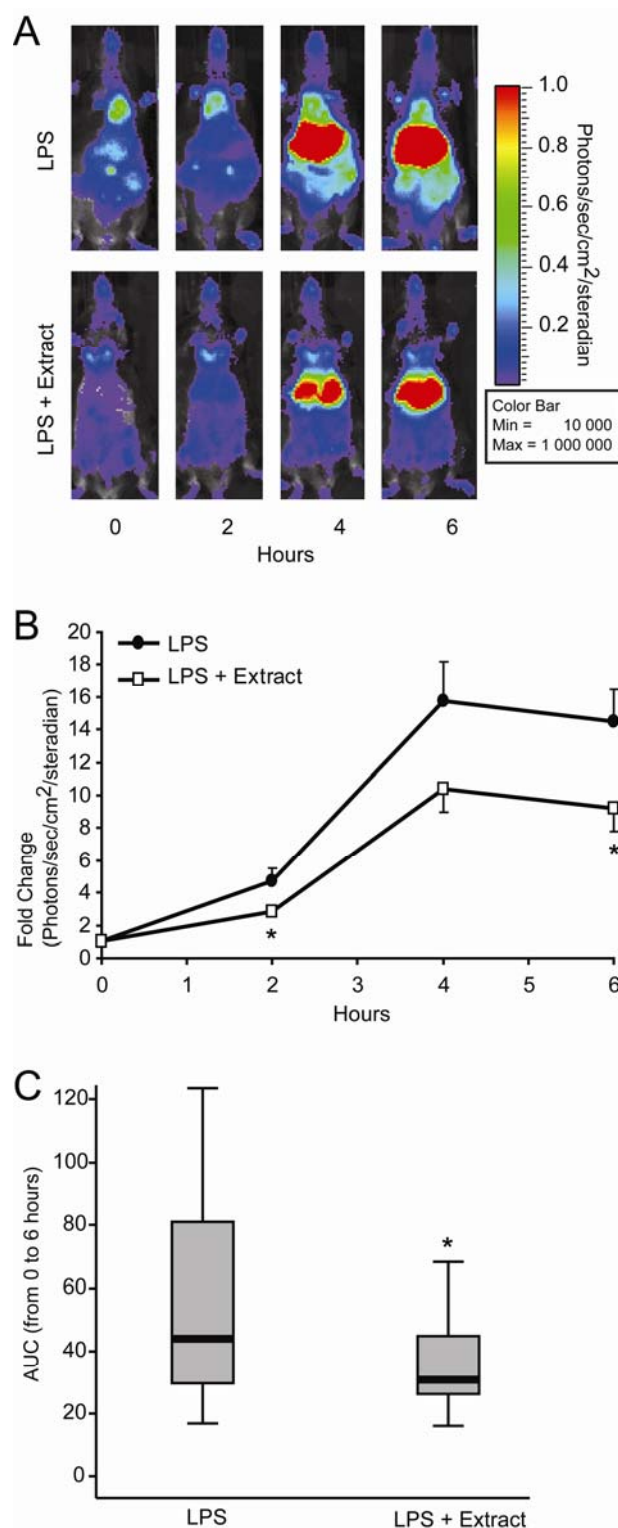


Figure 5.1. Effect of combination extract in transgenic NF- κ B reporter mice. *In vivo* imaging. Mice were given combination extract or control vehicle 3 h prior to LPS injection (0h). A) *In vivo* images of one representative mouse from each group. B) Mean \pm SEM fold change of luminescence (photons/sec/cm²/steradian) from the whole mouse minus head and extremities. C) The AUC was calculated for each mouse and compared between the treatment groups. The average photons emitted and AUC were compared with the Student's t-test. * = $p < 0.05$

Ex vivo imaging

Organ specific NF- κ B activation can not be detected by *in vivo* imaging. To evaluate differences organs (brain, lung, heart, thymus, spleen, kidney, liver, intestines, skin, muscle, white adipose tissue (WAT), uterus or testicle and epididymis) were excised and NF- κ B activation was measured by *ex vivo* imaging.

Ex vivo imaging after administration of the combination extract or vehicle control revealed a significant reduction of 0.53 fold ($p=0.028$) in the intestines of the extract group compared to the controls (**Figure 5.2 A**). Representative intestines from each group are shown in **figure 5.2 B**. The results for other organs (from the experiment performed within this thesis) are shown in appendix 1.

LUC assay

NF- κ B activity was measured in homogenates of organs (brain, lung, heart, thymus, spleen, kidney, liver, muscle, adipose tissue, uterus or testicle and epididymis).

The NF- κ B activity was reduced by 0.67 fold ($p=0.046$) in the liver, 0.66 fold ($p=0.007$) in the testicles and 0.48 fold ($p=0.021$) in the epididymis in the extract group compared to the controls. In the spleen, the NF- κ B activity was significantly increased in the extract group to 1.57 fold ($p=0.047$) of controls (**Figure 5.2 C**). There was a trend towards increase in the heart of the extract group compared to the control group, however not statistically significant. No differences between groups were detected in brain, thymus, lung, kidney, adipose tissue, muscle or uterus.

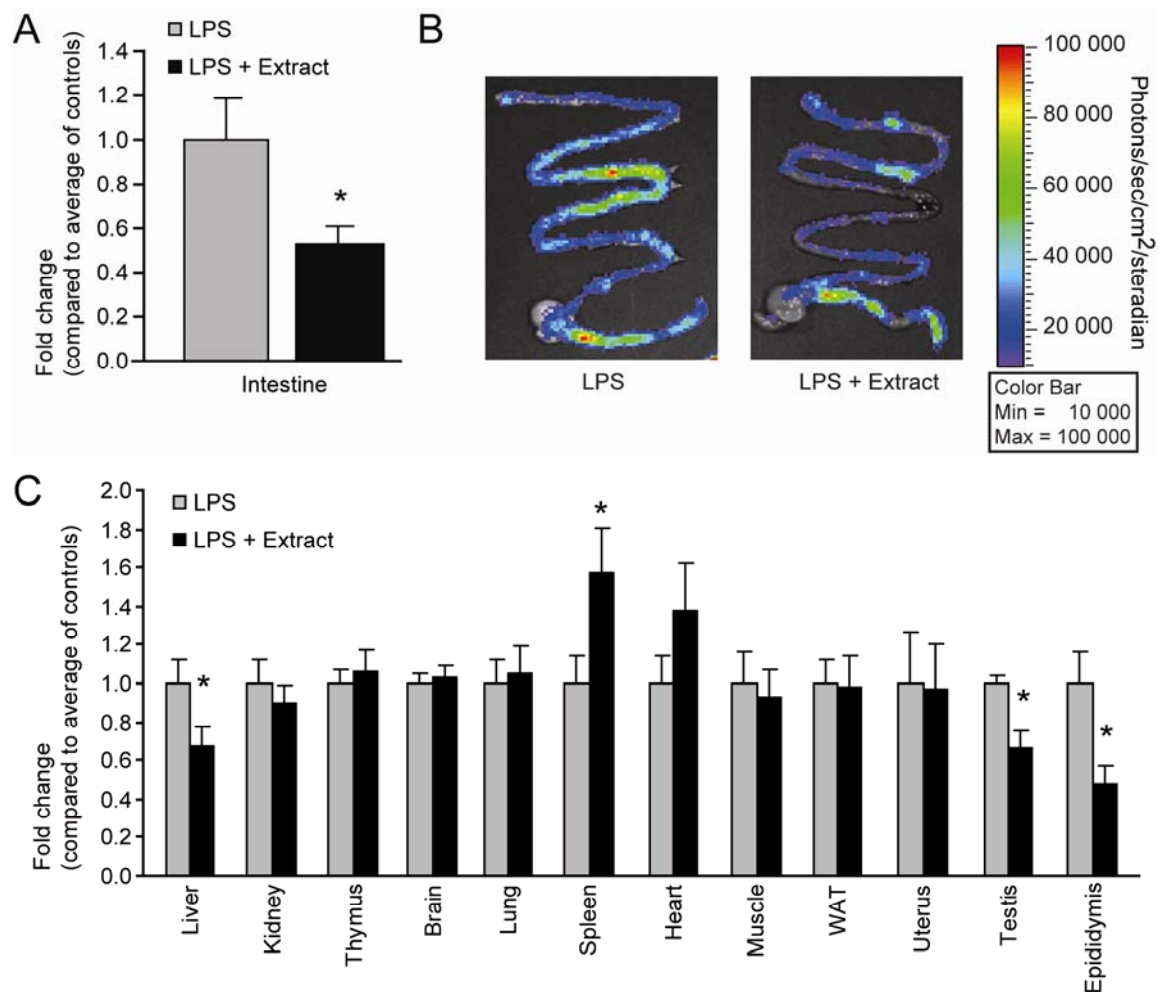


Figure 5.2. Effect of combination extract in transgenic NF- κ B reporter mice.

LPS induced luciferase activity in organs after administration of combination extract or control vehicle. Organs were excised and imaged 6 h post LPS injection, luminescence was measured by imaging in an IVIS 100 imaging system, and quantified using the “Living Image software”.

A: Quantification of emitted light from intestines. Bars represent mean fold change + SEM.

B: Representative intestines from one control mouse (left) and one combination extract mouse (right).

C: Quantification of emitted light from organs by LUC assay. Organs were excised 6 h post LPS injection, measured for luciferase activity and corrected for total protein content. Bars represent mean fold change + SEM. * = $p < 0.05$

5.2.2 Effect of single extracts; thyme, clove and walnut, on LPS-induced NF- κ B activity

Transgenic NF- κ B-luciferase reporter mice were given thyme/clove/walnut extract or control vehicle per os in a single bolus, 3 h prior to LPS injection (0h). NF- κ B activation was measured by *in vivo* imaging at -3, 0, 3, 6h, *ex vivo* imaging (at 6h) and LUC assay of individual organs. For all results the samples sizes are: Thyme: n=13 control, n=12 extract, clove: n=9 control, n=10 extract and walnut: n=9 control, n=8 extract.

In vivo imaging

In vivo imaging of mice given the combination extract showed inhibition of NF- κ B activation. Interestingly, this effect is not seen for any of the single extracts. One representative mouse from each treatment is shown in **figure 5.5**.

For the mice that received thyme extract (**Figure 5.5.A**), there are no differences when comparing AUC.

For clove (**Figure 5.5.B**), the mean AUC for the mice receiving extract is 1.87 fold increased in the clove extract group compared to controls, however this difference is not statistically significant ($p=0.243$).

The AUC of the walnut extract group (**Figure 5.5.C**) 1.85 fold increased compared to the control group. There is a trend towards a statistically significant difference between the treatments ($p=0.093$).

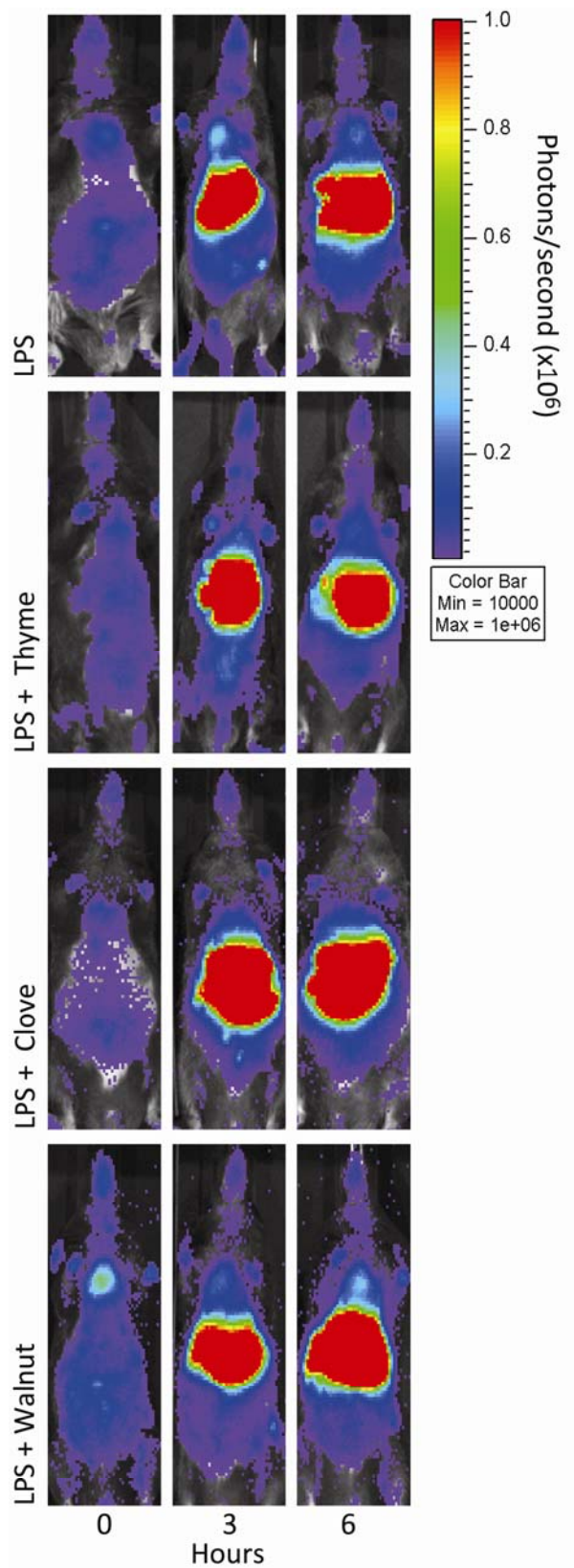


Figure 5.4. Effect of single extracts in transgenic NF- κ B reporter mice. *In vivo* imaging. One representative mouse from each group (control, thyme, clove and walnut) at 0, 3 and 6 hours is shown.

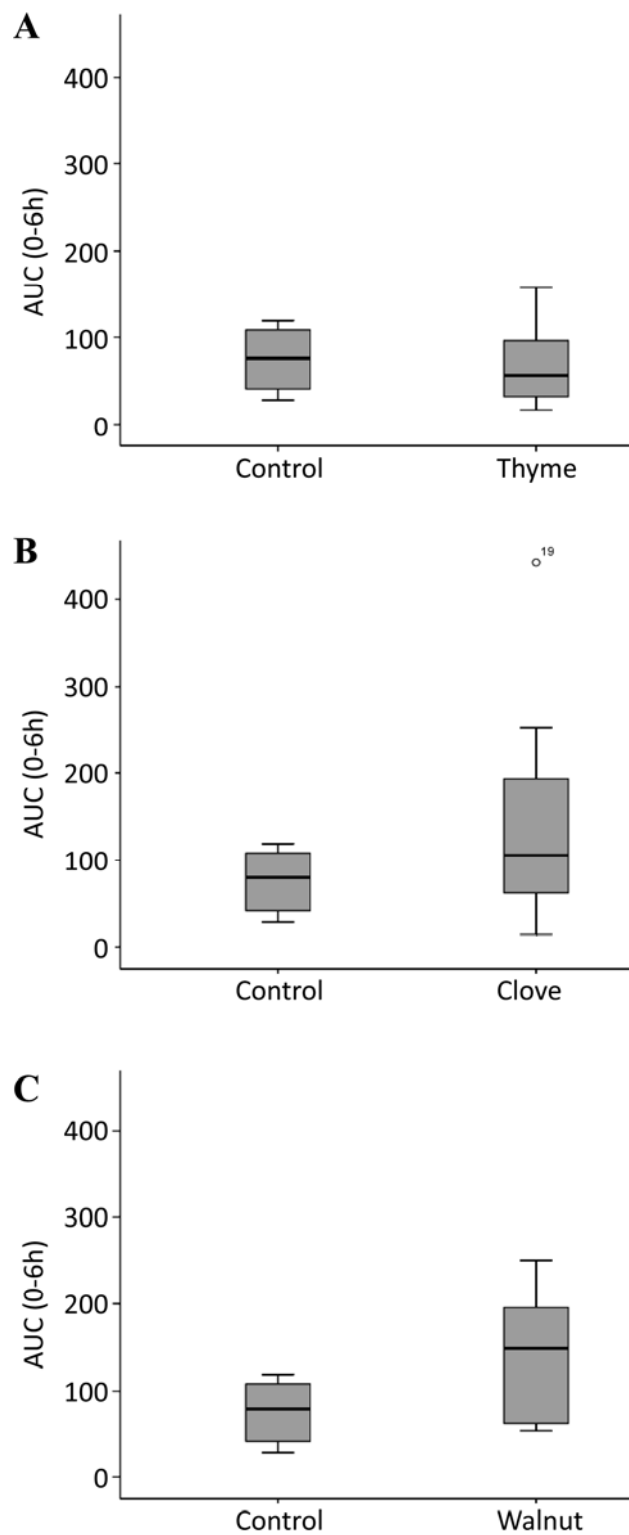


Figure 5.5. Effect of single extracts in transgenic NF- κ B reporter mice, - AUC.

Mice were given thyme (A), clove (B) or walnut (C) extract 3 h prior to LPS injection. The AUC was calculated for each mouse and compared between the treatment groups.

Looking at the time graphs (**Figure 5.6A**), there are no visible differences between the thyme and control group.

The mice that received clove (**Figure 5.6B**) have a seemingly higher activation of NF- κ B than their control mice, but this difference is not statistically significant at any time points. (At 3h, $p=0.278$, at 6 hours, $p=0.400$)

The walnut group (**Figure 5.6C**) also have a higher activation of NF- κ B compared to their control group. At 6 hours, there is a statistically significant difference of 104% higher overall NF- κ B activation in the walnut group compared to the control group ($p = 0.027$).

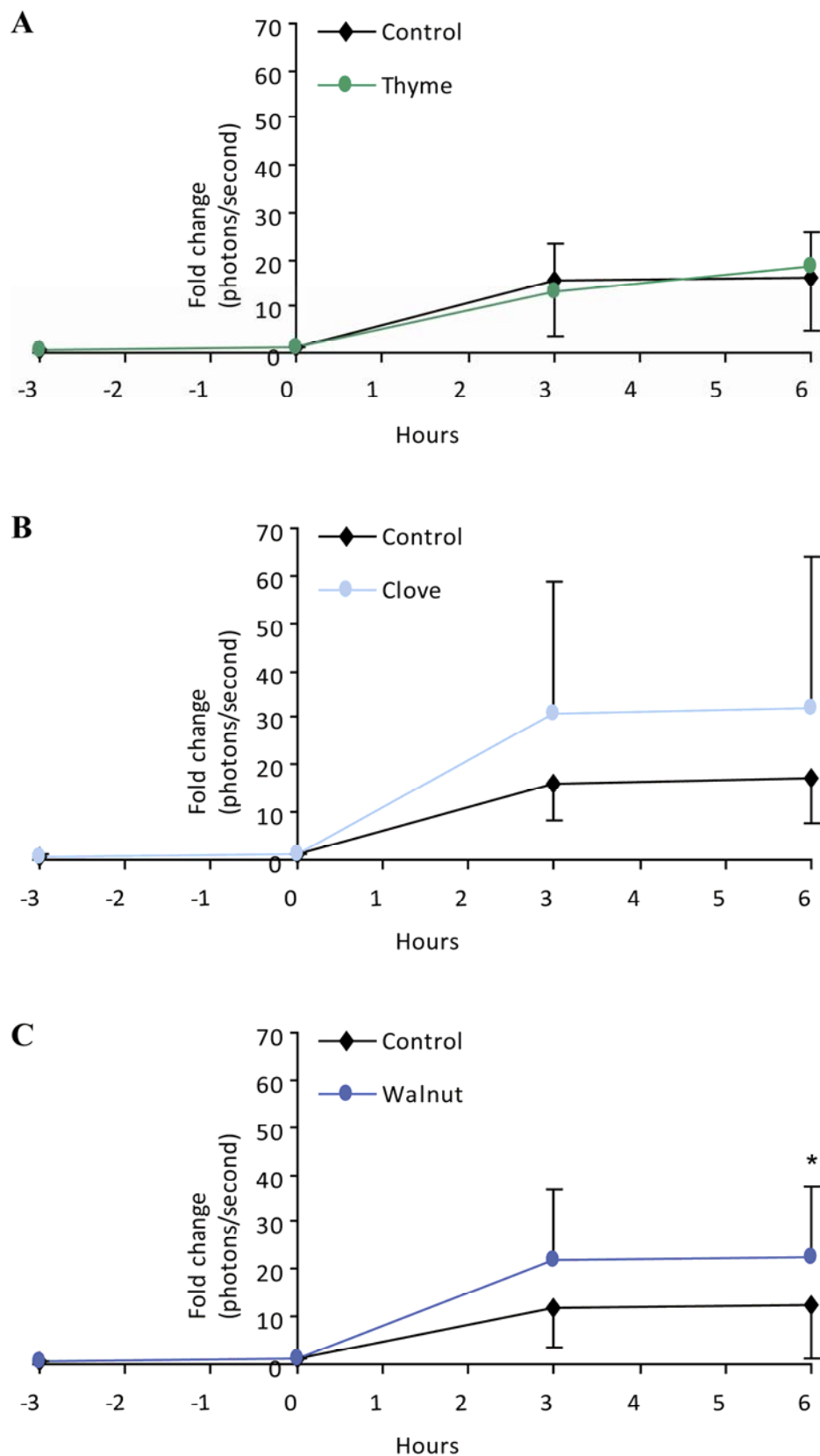


Figure 5.6. Effect of single plant extracts in transgenic NF- κ B reporter mice. *In vivo* imaging. Mice received thyme (A), clove (B) or walnut (C) extract 3 h prior to LPS injection and imaged at -3, 0, 3 and 6 hours. Time charts represent mean \pm SD of fold change (photons/second) from whole mice minus head and extremities. * = $p < 0.05$

Ex vivo imaging

Ex vivo imaging after administration of thyme (**Figure 5.8A**) showed that NF- κ B activity was increased 2.71 fold ($p=0.046$) in the epididymis in the extract group compared to the control group. No other organs showed significant change in NF- κ B activation with thyme treatment compared to control.

For the mice that were given clove extract (**Figure 5.8B**), there was a 0.54 fold ($p=0.035$) inhibition of LPS-induced NF- κ B activity in the spleen as compared to control mice. No other organs showed significant differences compared to controls after this treatment.

For the walnut treatment (**Figure 5.8C**), there was a tendency towards inhibition of LPS-induced NF- κ B activity in the spleen, with a 0.61 fold ($p=0.059$) reduction for walnut versus control-treated mice. This reduction is, however, not statistically significant. No other organs show any statistically significant differences compared to controls among the walnut treatment group.

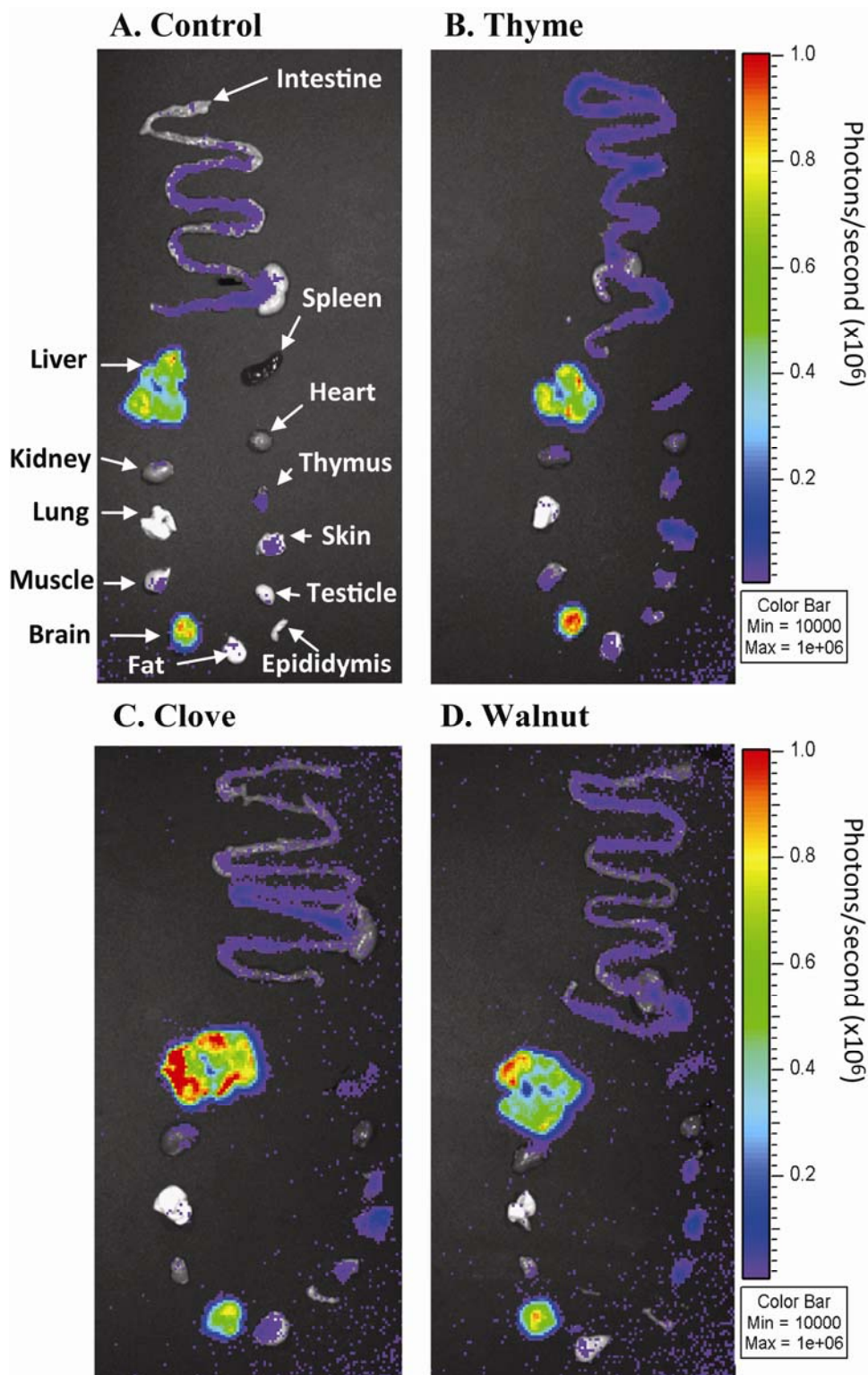


Figure 5.7. Effect of single plant extracts in transgenic NF- κ B reporter mice. *Ex vivo* imaging. LPS induced luciferase activity in organs after administration of control vehicle or thyme, clove or walnut extract. Organs were excised and luminescence was measured by imaging 6 h post LPS injection. Pictures show representative mice from single extract experiments. A: Control, B: Thyme, C: Clove, D: Walnut. The order of the organs is identical for all sets.

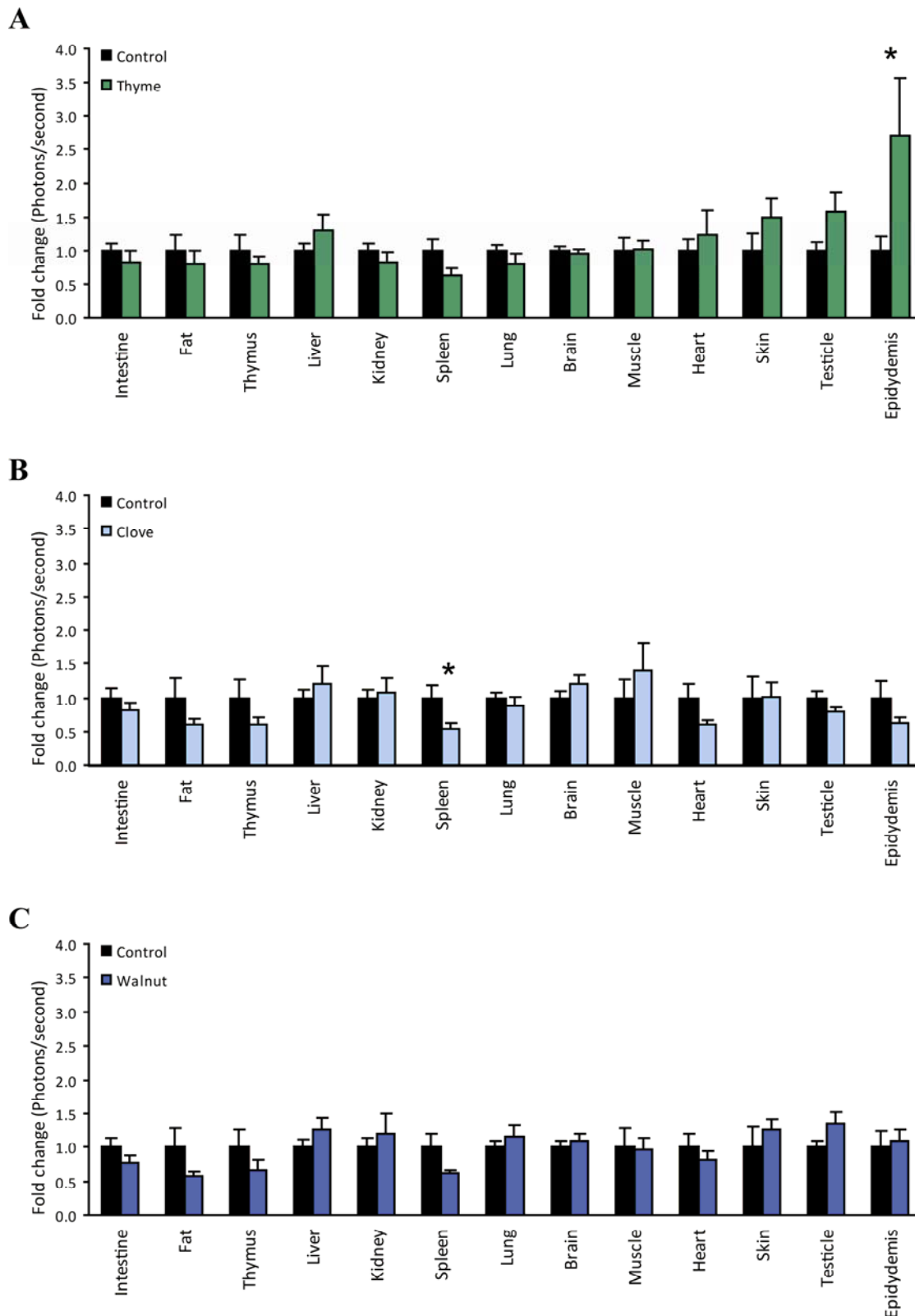


Figure 5.8. Effect of single plant extracts in transgenic NF- κ B reporter mice. Quantifications of *ex vivo* imaging. LPS induced luciferase activity in organs after administration of thyme (A), clove (B) or walnut (C) extract or control vehicle. Organs were excised 6 h post LPS injection and luminescence was measured by imaging in an IVIS 100 imaging system, and quantified using the “Living Image software”. Gray scale images were used for reference of position. Bars represent mean fold change + SEM. * = $p < 0.05$

LUC assay

NF- κ B activity was measured in homogenates of organs (brain, lung, heart, thymus, spleen, kidney, liver, muscle, adipose tissue, testicle and epididymis).

The NF- κ B activity of mice given thyme extract (**Figure 5.9A**) increased 1.82 fold ($p=0.046$) in the liver, 3.06 fold ($p=0.007$) in the muscle and 5.82 fold ($p<0.001$) in the epididymis, as compared to control mice.

The mice given clove extract (**Figure 5.9B**) showed a trend towards decreased NF- κ B activity in the spleen, with a 0.68 fold ($p=0.065$) reduction compared to controls. No other organs showed differences between the two treatments.

Walnut extract (**Figure 5.9C**) caused a 0.44 fold ($p=0.036$) reduction in NF- κ B activity in the heart compared to the control group. There is also a tendency towards inhibition in the kidney, however the difference is not statistically significant ($p=0.059$).

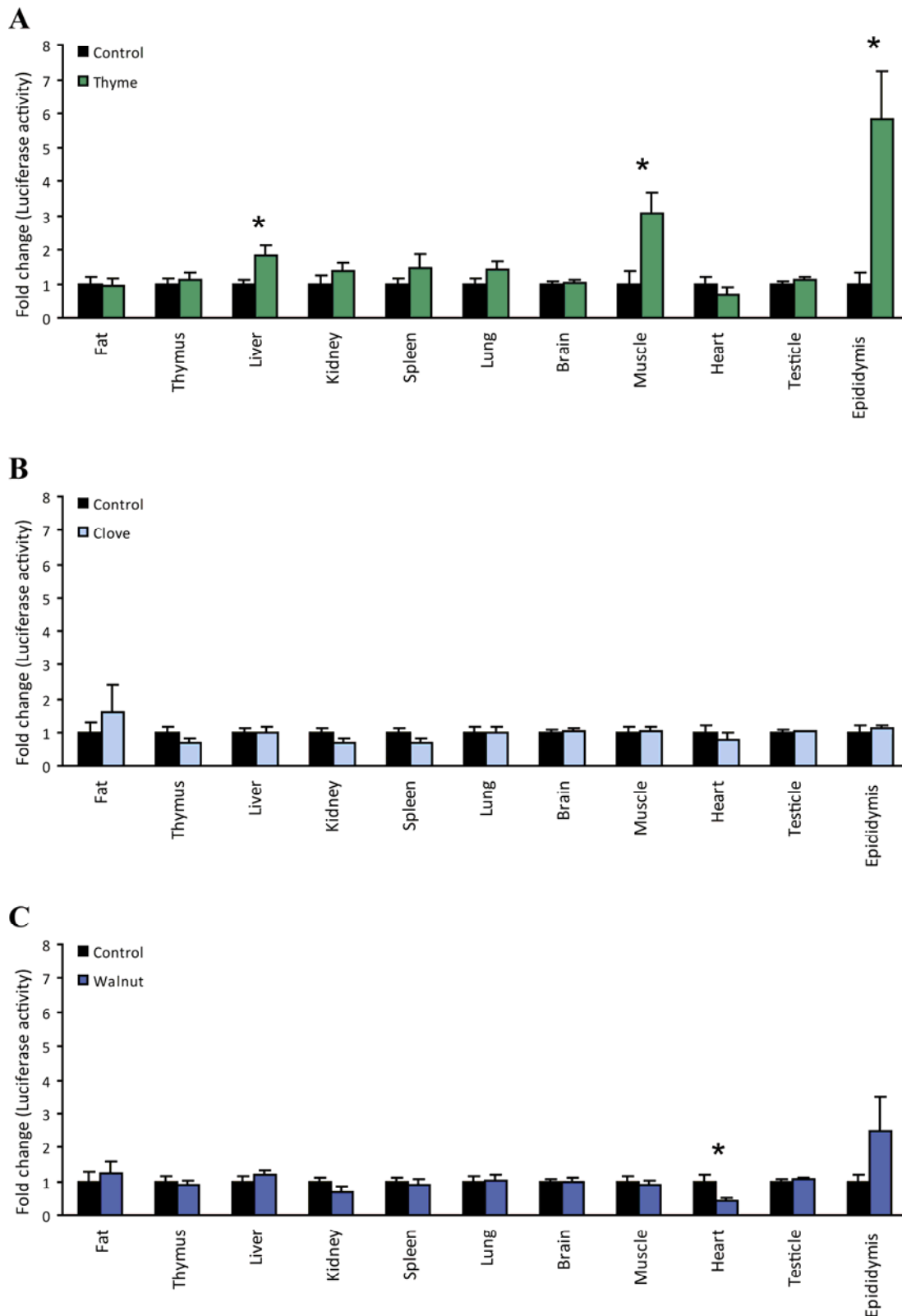


Figure 5.9. Effect of single plant extracts in transgenic NF- κ B reporter mice. LUC assay. LPS induced luciferase activity in organs after administration of thyme (A), clove (B) or walnut (C) extract. Organs were excised 6 h post LPS injection. Tissues were homogenised and luciferase activity was measured and corrected for total protein content of the sample. Bars represent mean fold change \pm SEM. * = $p < 0.05$

6. Discussion

6.1 Discussion of methods

6.1.1 The luciferase reporter system

The luciferase reporter system is based on production of luciferase from the LUC gene. Binding of NF- κ B dimers will lead to activation of the transcriptional machinery and production of luciferase mRNA. Luciferase is rapidly translated into functional proteins, with a half life of approximately two 2 hours, which makes this enzyme a favourable reporter for fluctuating NF- κ B activity (57). D-Luciferin is injected intraperitonally, providing a rapid distribution to all organs. Where luciferase is present, D-luciferin is oxidised to oxyluciferin, and light is emitted. D-Luciferin is given in excess to ensure that luciferase produced by NF- κ B activation, and not the substrate, is the rate limiting factor in the enzyme reaction.

The luciferase reporter system can detect low levels of gene expression, and the light emitted is largely correlated with the amount of luciferase present (57).

The measurements are, however, compromised by a variable level of absorption of photons in different tissues. Haemoglobin absorbs light emitted from the luciferase/D-luciferin reaction, which affects measurements especially in blood rich organs. Blood rich organs such as the kidneys, lungs and heart emit little light, which can lead to underestimation of NF- κ B activation in these organs compared to other organs that contain less blood (50). This may, at least in part, explain the relatively low luminescence from kidneys, lungs and heart that can be seen with the naked eye on the *ex vivo* images in “Results”, Figure 5.7.

Furthermore, there is a limitation as to which extent the luminescence can penetrate tissues. When imaging *in vivo*, luminescence from the deeper layers of tissue is less likely to be detected, and a disproportionate amount of the signal would come from the organs close to the surface, such as skin and muscle (57). This limitation will to some

degree also apply to *ex vivo* imaging, due to the relative difference in dimensions of the organs. It is reasonable to believe that in a small organ like the thymus, most of the luminescence occurring will be detected, whereas in larger organs, such as the liver, a smaller portion of total luminescence may be detected. However, as this will apply equally to all the animals used in the experiment, the relative differences measured will reflect a real difference in gene expression.

Transgenic reporter mice and the luciferase reporter

Methods involving *in vivo* imaging of model organisms have become valuable tools to study real time gene expression. These rapid and non-invasive assays allow multiple measurements within one animal. Several methods are used to image gene expression, among which positron emission tomography (PET), magnetic resonance imaging (MRI) as well as measuring green fluorescent protein, near infrared protein and luciferase are applied most frequently (57).

In this project, transgenic mice expressing the LUC gene were used to measure LPS-induced NF- κ B activity and its possible modulation by plant extracts. When these mice are generated, multiple copies of a DNA construct, containing three κ B binding sites upstream of the LUC gene, are incorporated randomly into the mouse genome. Activation of the LUC gene is dependent both on binding of the NF- κ B dimer as well as the dimer's ability to activate transcription, which makes luciferase a reliable reporter of this specific transcription factor.

A disadvantage of the method is the randomness of genomic positioning and the unknown copy number of constructs inserted, as these factors will affect the transcription rate. More copies will give a higher expression level. For reporting purposes this can be beneficial, as an increased expression can increase the sensitivity of the method. However, differences in integration numbers between mouse lines can lead to large variations between experiments. Epigenetic differences may influence transcription levels, as insertion in heterochromatic areas of DNA can reduce or block transcriptional activation. The transcriptional activity rate of neighbouring DNA areas

can also influence the expression level, as certain DNA areas are more transcriptionally active than others.

The design of the *in vivo* experiments can circumvent the above mentioned limitations of the luciferase reporter method to a certain extent. Firstly, all the mice used in this project are descendants of the same line; hence the copy numbers between the individuals are identical. Nevertheless, epigenetic variety may occur. Secondly, when luminescence is measured, the fold change in NF- κ B activity from the time of LPS injection is calculated for all animals at all time points. This means that each animal serve as an internal control. As long as there are no differences concerning basal NF- κ B activity between the groups prior to treatment, a significant difference after treatment will reflect an effect caused by the treatment. Statistical tests, for the difference of the mean of the control and the extract group for each experiment, revealed no significant differences between the groups prior to treatment.

LUC assay

In addition to *in vivo* and *ex vivo* imaging, LUC assays were performed on all excised organs, except intestine and skin. In the LUC assay, the organs are homogenised in a lysis buffer solution, to compensate for the luminescence' lacking ability to penetrate tissues. This will release the luciferase produced within the cells, and make it accessible to react with D-luciferin in the assay solution. The snap-freezing of the organs after *ex vivo* imaging stops all chemical reactions in the cells and preserves the luciferase within the tissue.

6.1.2 FRAP measurements

There are several ways of measuring total antioxidant capacity in food extracts. Corral-Aguayo et al. (58) measured total antioxidant capacity of food materials using six different assays: 2,2'-diphenyl-1-picrylhydrazyl (DPPH), N,N-dimethyl-p-phenylendiamine (DMPD), ferric reducing ability of plasma (FRAP), oxygen radical absorbance capacity (ORAC), Trolox equivalent antioxidant capacity (TEAC) and

total oxidant scavenging capacity (TOSC), and found that there was a strong correlation between the total phenolics measured for all methods.

The FRAP method is well established in our laboratory, and had been used in earlier, related work, which allowed comparisons with previous measurements. The FRAP method is rapid and straightforward, linearity is maintained over a wide range and it gives a direct measure of the reducing compounds in a sample (59).

The combination extract used in this work was prepared and measured for FRAP in connection with earlier experiments (Table 5.1.). Extracts of thyme, clove and walnut have previously been determined for FRAP by Paur et al. (34), to values of 21.0, 141.5 and 7.7 mmol/100 g, respectively. In this project, the extract of thyme and walnut had higher FRAP values, whereas the clove extract had a lower FRAP value than previously measured. Differences in amount of extraction fluid between extract preparations could explain some of the differences. The ratio between sample and solvent can affect the total content of phytochemicals extracted from the foods (60). The amount of solvent used in the extraction will also affect the time of evaporation under nitrogen, a preparation step that can cause phytochemicals to be lost in the vapour.

When original plant components were assayed, Halvorsen et al. (59) found a FRAP value of 20.97 mmol/100 g for walnut. Dragland et al. (61) measured three types of thyme, with FRAP values ranging from 45.4-95.0 mmol/100g. They also determined clove to contain 465.3 mmol/100 g.

In addition to preparation procedures, variations may be explained based on the species, growing conditions and production. Botanic varieties between producers, different countries of origin, the time and conditions of cultivation and storage before and during sale could influence the amount of phytochemicals in the plants (59).

The FRAP analysis' were performed to confirm that the extracts had turned out rich in phytochemicals. The total antioxidant capacity measured by FRAP reported a high content of reducing compounds in the extracts used in this project. Several studies on

similar types of food extracts have established a strong correlation between total phenolic content and total antioxidant capacity (17;62;63). It is reasonable to believe that a considerable share of the total antioxidant capacity is owed to phenolics in the extracts.

6.2 General discussion

A diet rich in plant-based foods can protect against many degenerative diseases, and this protection could, at least partly, be ascribed to bioactive phytochemicals and their influence on gene regulation. Plants contain thousands of chemical substances, and the possible interactions between the compounds found within one plant, not to mention the combinations provided from a varied, plant-based diet, are almost impossible to predict. Cellular signalling cascades of cells interact in complex ways. An effect on one specific cellular pathway observed *in vitro* might be altered by interacting cellular pathways *in vivo*.

The link between aberrant NF- κ B activation and diseases, especially some types of cancer, has made this transcription factor a popular target for research towards both prevention and treatment of these diseases. Research about NF- κ B modulating properties of phytochemicals have largely been focusing on single compounds. From a nutritionist's point of view, it is of great interest to study how whole foods can contribute to prevention of these diseases. Accordingly, the focus of this thesis is on the effect of extracts from whole foods. Also, most of the NF- κ B modulating properties of phytochemicals have been demonstrated *in vitro*. *In vivo* data on NF- κ B modulation by plant extracts are scarce. The use of transgenic luciferase reporter mice brings research about NF- κ B modulations by phytochemicals to a new level.

The work in this thesis is part of a larger project in the Rune Blomhoff research group, in which several plant-food extracts have previously been found to modulate NF- κ B activation in U937 3 \times κ B-LUC cells (34). A combination extract made from five of the most potent inhibitors (coffee, thyme, oregano, clove and walnut) from this previous

screening was also found to synergistically inhibit LPS-induced NF- κ B activity in the U937 3 \times κ B-LUC cells (64).

Based on these findings, we proceeded to study the effect of the combination extract *in vivo* in transgenic reporter mice. We also decided to test whether the single components of the combination extract could modulate NF- κ B activation *in vivo* one by one. The single plants chosen were among the most potent NF- κ B inhibitors in *in vitro* experiments, and they were all parts of the combination extract. Coffee extract had previously been tested *in vivo* by Paur and Balstad et al. (47), with significant reduction in NF- κ B activation as the result. Oregano and thyme had the most overlapping phytochemical profiles of the four remaining candidates, and thus we decided to leave out one of these.

The results from the combination extract experiments showed significant inhibition of LPS-induced NF- κ B activity for mice treated with combination extract compared to those receiving control vehicle only. The extract group had a 35 % lower activation based on total AUC (0-6 h).

The *in vivo* experiments with single extracts did not confirm the inhibitory effects on NF- κ B activity seen *in vitro*. Thyme extract did not modulate overall NF- κ B activation *in vivo* in our experiment, and walnut showed an increase in NF- κ B activity. Clove extract showed a trend towards increase in NF- κ B activity.

Both the combination extract and the single extracts caused organ specific alterations in LPS-induced NF- κ B activity, and the effects from the combination extract and the single extracts are somewhat contradictory. **Table 6.1** summarises the significant differences and possible trends of NF- κ B modulations by plant extracts tested in this project.

Table 6.1. Overview of the main findings from *in vivo* experiments with combination and single extracts.

Treatment	Reduced NF-κB activity	Increased NF-κB activity
Overall NF-κB activity		
Combination extract	35 % lower AUC (0-6 h)	
Walnut extract		104 % higher fold change at 6 h
Organ specific NF-κB activity		
Combination extract	Intestines, liver, testicle, epididymis	Spleen
Thyme extract		Liver, muscle, epididymis
Clove extract	Spleen	
Walnut extract	Heart	
Organ specific NF-κB activity - trends		
Combination extract		Heart
Walnut	Kidney, spleen	

6.2.1 Phytochemical profile and bioactivity of the plants used in this project

To identify the phytochemicals' molecular mechanisms of action, a comprehensive analysis of the extracts would be necessary. This can be achieved by chromatographic methods, e.g. High Performance Liquid Chromatography (HPLC). The phytochemical profiles of the extracts used in this project are not known, but from analyses performed in other laboratories, we can assume a similar phytochemical profile of our extracts.

Coffee (*Coffea Arabica*) contain a large number of phytochemicals, from which caffeic acid and chlorogenic acid constitute the major part of the phenolics (66). Coffee extract (30 mg/ml) inhibited LPS-induced NF-κB activity *in vitro* by 89 % (34). This inhibitory effect was subsequently reproduced *in vivo* (47).

Thyme (*Thymus vulgaris*) has been reported to have a total phenolic content of 4.52 g per 100 g, and the major phenolic compounds are caffeic acid, *p*-coumaric acid, rosmarinic acid, gallic acid and thymol (17). Thyme extract resulted in a 88.7 % reduction in LPS-induced NF-κB activity in U937 3×κB LUC cells (34).

Walnuts, the seeds of *Juglans regia*, are rich in poly-unsaturated fatty acids (PUFA), which make them susceptible to oxidation. However, the walnut seeds are stable against oxidation, possibly due to their high content of phenolics with antioxidant capacity (67). The major fraction of phenolics are located in the pellicles, (the protective, brown skin that covers the kernel), while the seed itself contains relatively small amounts of phenolic compounds. Among the phenolics identified from walnuts are: Chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid and ellagic acid (68). Paur et al. (34) reported that walnut extract (30mg/ml) gave a 63.8 % reduction in LPS-induced NF- κ B activity in U937 3 \times κ B LUC cells.

Oregano, *Origanum vulgare*, contains approximately 10.17 g phenolics per 100 g, and caffeic acid, *p*-coumaric acid, caffeoyl derivatives, rosmarinic acid, carvacrol and kaempferol are among the major contributors (17). Oregano was the most potent inhibitor of NF- κ B activation by LPS in *in vitro* studies by Paur et al. (34), causing a 91.4 % reduction compared to control.

Clove, *Syzygium aromaticum*, has had its total phenolic content measured to 14.38 g per 100 g, with a substantial proportion made up of the phenolic volatile oil eugenol. Clove also contains quercetin (17). Clove extract reduced LPS-induced NF- κ B activity with 87.8 % *in vitro* (34). Aggarwal and Shishodia (37) showed that eugenol and isoeugenol inhibit NF- κ B activation by suppressing I κ B α degradation *in vitro*.

6.2.2 Effect of plant extracts on LPS-induced NF- κ B activity

In vivo imaging

The mice receiving the combination extract showed a 35% reduction in LPS-induced NF- κ B activation compared to controls, calculated as total AUC, and the overall difference between the treatment groups is statistically significant.

The combination extract is likely to contain a large number of the bioactive substances present in coffee, thyme, oregano, clove and walnuts, which could modulate NF- κ B signalling in additive, synergistic and antagonistic ways.

The *in vivo* imaging after administration of single extracts show a different effect on NF- κ B compared to the combination extract. The 1.87 and 1.85 fold increases in the clove and walnut group, respectively, could indicate that these extracts, when administered alone, have an opposite effect on overall NF- κ B modulation compared to the combination extract.

An important factor to consider is the amount of phytochemicals provided by the extracts. The amounts given in one single dose of combination extract would convert to more than 100 cups of coffee, 1.5 kg walnuts and 150 grams of thyme, oregano and clove, respectively, for a person weighing 75 kg, provided that all the phytochemicals from the foods were extracted. Such a phytochemical dose could not be provided by any normal diet, and might be considered pharmacological. Still, *in vivo* effects caused by the plant extracts could be starting points for further research.

The doses of both clove and thyme extracts were ten times higher than in the combination extract. The mice were given the maximum concentration possible to increase the possibility of observing an effect. The dose could have been lowered; however, as the mice receiving the combination extract got a higher total dose of phytochemicals than those receiving single extracts (Table 4.2), testing the single extracts with a maximum dose seemed rational. If such a dose exceeds a beneficial amount of phytochemicals, thereby provoking an inflammatory reaction in the animals, the increased NF- κ B activity observed from the clove extract could be the result of a xenobiotic overload.

Walnuts are generally considered anti-inflammatory, due to their high content of essential PUFAs. The phenolic ellagic acid has also shown potent anti-inflammatory effects in experimental studies (69). In this project, however, walnut extract was the only single extract that gave a significant increase in LPS-induced NF- κ B activity.

The walnut dose was the same as in the combination extract. *In vitro*; walnuts showed the strongest effect among the foods tested, to both induce basal activity and inhibit LPS-induced activity of NF- κ B (34). If this also applies to mice, the LPS-induced NF- κ B activity could come on top of an increase in basal activity, possibly explaining some of the increase in NF- κ B activity measured.

Ros (69) evaluated nuts in the context of cardiovascular disease, and put forward a suggestion that oxidation-prone PUFAs from walnuts could enhance oxidation, unless counterbalanced by antioxidants. The role of oxidative stress in NF- κ B activation is much disputed, even though some scientific publications state the redox sensitivity of NF- κ B as a matter of course. Early hypothesis' predicted NF- κ B to be redox sensitive (70), however opinions among scientists in the field seem to be split. Provided that NF- κ B is a redox sensitive transcription factor, this argument could fit the observation that walnut alone led to increase in NF- κ B activity, whereas walnuts in combination with other phytochemical-rich foods attenuated the response.

A large share of the activity measured by *in vivo* imaging is probably originating from the liver. The livers of mice comprise a large percentage of the body weight compared to humans. The intense light signal seen by *in vivo* imaging (**Figure 5.1A** and **Figure 5.4**) after LPS injection, at the upper part of the abdominal area, seems to correspond with the position and size of the liver. High activity in the liver measured by both *ex vivo* imaging and the LUC assay supports this interpretation. The absolute NF- κ B activity in the liver (adjusted for protein content) is among the highest of all organs, and combined with the large size, the liver contributes considerably more than any other organ to the overall NF- κ B measured *in vivo*.

Ex vivo imaging and LUC assay

The analyses of organs by *ex vivo* imaging and the LUC assay can further specify the source of NF- κ B activation measured by *in vivo* imaging. Altered NF- κ B activity in organs could reflect bioavailability and specific tissue distribution of phytochemicals and their metabolites. In cases where phytochemicals are metabolised and conjugated

in the intestine and the liver, other organs could be exposed to metabolites of the original compound. These differences in tissue exposure to phytochemical metabolites could cause organ specific responses on a major signalling pathway as NF- κ B.

Knowledge of the tissue distribution of phytochemicals present in our extract is scarce, and data on phytochemical metabolomics is limited. However, a few studies have monitored radio-labelled phytochemicals after oral administration. A study on resveratrol distribution in mouse tissue revealed a preferential presence in the organs and biological liquids of absorption and elimination (stomach, liver, kidney, intestine, bile, urine) (71). Suganuma et al. (72) investigated the tissue distribution of epigallocatechingallate (EGCG) after oral administration in mice, and found EGCG or its derivatives in a wide range of organs; intestines, lung, liver, pancreas, skin, kidney, brain, uterus, ovary and testicles. Mullen et al. (73) investigated metabolism and tissue distribution of [2- 14 C]quercetin-4'-glucoside, and found that the majority was rapidly conjugated in the intestines and incorporated into metabolites. There was limited absorption of quercetin into the blood stream and organs.

The observed inhibition of NF- κ B activity in specific organs indicates that bioactive compounds from the combination extract have been absorbed, and that these compounds or their metabolites modulate NF- κ B signalling in the respective organs. Consistent with the observations done by Vitrac et al. (71), we found alterations in NF- κ B signalling in organs of absorption and elimination; such as the intestines, liver and possibly also the kidneys. *Ex vivo* imaging reported a statistically significant inhibition of LPS-induced NF- κ B activity in the intestines of the mice receiving combination extract. The LUC assay on tissue homogenates revealed statistically significant inhibition in the liver from the same treatment. High concentrations of phytochemicals in the intestines and liver are expected, as these organs are the main sites of phytochemical metabolism. Bioactive properties of phytochemicals in the digestive tract are supported by results from animal studies. Bobe et al. (74) found a reduction in tumour development after treatment with anthocyanins in a mural intestinal cancer model. Kim et al. (75) found that 3,3-Diindolylmethane (DIM),

an *in vivo* metabolite from cruciferous vegetables, significantly reduced inflammation and improved pathology from colitis, as well as reduced the number of tumours from colitis-associated colon cancer in mice. They also found that treatment with DIM reduced the NF- κ B binding in the nuclei of colonic tissue cells, and further mechanistic *in vitro* experiments confirmed that DIM blocked I κ B degradation and thereby nuclear translocation of NF- κ B.

The inhibition of NF- κ B activity in the liver after treatment with the combination extracts is supported by other studies that have revealed liver specific effects of phytochemical-rich foods. Shimoda et al. (67) found that orally administered phenolics from walnut reduced carbon tetrachloride (CCl₄)-induced liver injury in mice. They also found that activation of NF- κ B is involved in the induction of liver damage in this model. Botsoglou et al. (76) investigated the effect of long term oregano feeding on the alleviation of CCL₄-induced liver damage in rats, and found that oregano significantly reduced the circulating markers of liver damage post treatment. Sasaki et al. (77) found that both thyme and phenolic compounds from thyme (thymol and carvacrol) were able to induce production of cytoprotective phase I and II enzymes in the liver using a mouse model. Two coffee specific compounds, cafestol and kahweol, have shown anti-carcinogenic properties in a rodent model by modulating the activity of phase I and II enzymes (78).

From the *ex vivo* imaging (**Figure 5.6**), all single extracts seem to cause a somewhat increased NF- κ B activity in the liver, and for the thyme treatment, the LUC assay confirms this trend by showing a significant increase in NF- κ B activity. The overall contribution of the liver to the NF- κ B activity measured (as discussed previously) could explain some of the increased signal *in vivo* after treatment with walnut and possibly also clove, even though we did not find significant differences in the liver for these treatments.

Interestingly, we observed effects also in the testicle and the epididymis. The combination extract inhibited NF- κ B activation in both these organs, whereas the mice receiving thyme extract showed a significant increase in the NF- κ B activity of the

epididymis. Clearly, the epididymis is sensitive to changes caused by phytochemicals. LPS injection induces ROS formation in cells (21;79), and DNA is susceptible to damage by ROS. The reproductive organs might be especially sensitive to changes in cellular ROS, as the testicle produces sperm (DNA is continually being replicated), and the epididymis matures the newly produced sperm.

Treatment with the combination extract caused increase in NF- κ B activity in the spleen. The clove extract caused a significant reduction of NF- κ B activity in the spleen measured by *ex vivo* imaging; however, this was not supported by the LUC assay which is generally a more reproducible method for most tissues. Still, this adds to the observation that the combination extract seems to produce effects opposite of the single extracts.

We found a significant increase in NF- κ B activity at 6 hours in the walnut extract group measured by *in vivo* imaging. However, no organs showed a significant increased activity measured by *ex vivo* imaging or LUC assay. The overall increased NF- κ B activity observed by *in vivo* imaging is probably caused by partial contributions of several organs emitting luminescence, though not showing statistically significant differences in our assays.

A possible cross-talk between NF- κ B and the cytoprotective proteins, which are induced by the transcription factor Nrf2, has been suggested (80;81).

In vitro and *in vivo* experiments have revealed the dual ability of some phytochemicals (curcumin, resveratrol, EGCG, caffeic acid phenethyl ester (CAPE) and isothiocyanates, e.g. sulforaphane) and coffee to inhibit NF- κ B activation and induce Nrf2 (5). Activation of the Keap1/Nrf2 pathway will result in transcriptional activation of several cytoprotective proteins involved both in the xenobiotic metabolism and generation of endogenous antioxidants (3). If the combination extract activates cytoprotective gene transcription, the subsequent cellular stress by LPS is possibly lowered in the extract group, leading to an attenuated NF- κ B activation. This observation fits the “hormesis theory”, i.e. phytochemicals can prepare the cells for stress by inducing the expression of cytoprotective proteins. According to the theory,

the molecular responses triggered by stress-inducing compounds do not only protect against the same compound, but also less specific stressors. Possibly, such responses can aid more general cellular stress conditions, such as oxidative, metabolic or thermal stress (32).

6.2.3 Single extracts versus combination extract

The effects observed in the combination extract experiment are difficult to interpret in context of the single extract experiments. As coffee extract alone proved to have an inhibitory effect on NF- κ B *in vivo* (47), the coffee in the combination extract could possibly counteract or exceed the effect caused by the walnut or clove extract. The combination extract could also produce a very different response, due to interactions between the phytochemicals provided. Future analysis of the extracts' bioactive compounds, and the distribution of phytochemicals and their metabolites in tissues, could provide new insight into these somewhat contradictory results.

One important factor concerning the experiments with single plant extracts is the sample size. The combination extract experiment was repeated three times before the sample size was sufficient to yield statistically significant differences, due to rather large inter-individual variations. The results from single extract experiments need to be reproduced before conclusions about the effect on NF- κ B activation can be drawn; however, for the combination extract, all the individual experiments *in vivo* were supporting results from *in vitro*.

In vitro studies traditionally use cells from only one tissue. In live animals, the phytochemicals are absorbed, conjugated and distributed to tissues to various extents. Hence, the phytochemicals may influence several tissues, and in different ways. The fact that thyme, clove and walnut extracts produce different effects *in vitro* and *in vivo*, illustrates the complexity of the interplay between phytochemicals and physiological effects in a living organism. If the hormesis theory applies to our experiments, the doses we have used might have been too high, possibly causing an adverse effect instead of an inhibiting effect. Examples of possibly adverse effects of phytochemical

supplements were seen in two studies on β -carotene and vitamin A, and β -carotene and vitamin E, respectively. These clinical trials were based on epidemiological studies indicating that dietary intake of carotenoids is linked to a reduced risk of developing chronic diseases. Supplementation of carotenoids in high doses, however, increased the prevalence of lung cancer and increased mortality from heart disease in smokers, and both trials were discontinued (82;83). This could suggest that positive health effects arise from normal dietary levels, whereas abnormally high amounts can have detrimental effects. Besides, even though a diet rich in β -carotene is protective against disease, β -carotene is not necessarily the protective agent. This might also be the case for other presumably bioactive dietary compounds.

In nutritional science, this exemplifies the concern expressed by several scientists on the overwhelming promotion and use of antioxidant supplements. The antioxidant supplement industry might promote doses that exceed the normal, if not toxic threshold, potentially causing harm rather than protection. A substantial amount of evidence supports that a diet rich in plant foods protects against disease and prolong life expectancy. Exposure to small, continuous doses of phytochemicals provided by natural foods might be one part of the explanation for the observed protection.

7. Conclusion

The object of this Master project was to follow up and continue previous work performed in the Rune Blomhoff research group on NF- κ B modulating properties of phytochemical-rich plants.

The treatment of transgenic reporter mice with combination extract showed an overall significant inhibition of LPS-induced NF- κ B activity. Inhibition in the extract group at 2 and 6 h were 40 % and 36 %, respectively. The AUC (0-6 h) was 35 % lower in the extract treatment group compared to the control group. The combination extract also had the ability to modulate NF- κ B activation in an organ specific manner. Activation in intestines, liver, testicles and epididymis was significantly inhibited in the extract group, whereas the activity in the spleen was significantly increased.

The *in vivo* treatments with single extracts (thyme, clove and walnut) revealed a higher NF- κ B activity when the mice were treated with walnut extract, and possibly also with clove extract, than with LPS only. At 6 h, the NF- κ B activation was 104 % higher in the walnut group compared to the control group, a statistically significant difference. We found organ specific NF- κ B modulation also by single food extracts, particularly in the reproductive organs (testicle, epididymis) and in the spleen; however, the respective organs seem to respond in an opposite way after treatment with single extracts compared to the combination extract treatment.

The observation that effects of phytochemical-rich plant extracts produce a different effect on NF- κ B modulation *in vivo* than *in vitro*, illustrates the complexity of the interplay between phytochemicals and physiological effects in a living organism. As dietary plants contain an abundance of phytochemicals, a multitude of possible mechanisms of action could cause the effects observed.

The results from this project have shown that dietary plants can influence NF- κ B signalling. Further work should focus on revealing the bioactive substances in the extracts, and the mechanisms behind their NF- κ B modulating properties.

8. Reference list

1. Muller M, Kersten S. Nutrigenomics: goals and strategies. *Nat Rev Genet* 2003;4:315-22.
2. WHO. The world health report. 2003. World Health Organisation.
Ref Type: Data File
3. Dinkova-Kostova AT, Talalay P. Direct and indirect antioxidant properties of inducers of cytoprotective proteins. *Mol Nutr Food Res* 2008;52 Suppl 1:S128-S138.
4. Scalbert A, Manach C, Morand C, Remesy C, Jimenez L. Dietary polyphenols and the prevention of diseases. *Crit Rev Food Sci Nutr* 2005;45:287-306.
5. Surh YJ, Kundu JK, Na HK, Lee JS. Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. *J Nutr* 2005;135:2993S-3001S.
6. Szent Gyorgyi A, Rusznyak S. Vitamin P: Flavonols as Vitamins. *Nature* 1936;136.
7. Liu RH. Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J Nutr* 2004;134:3479S-85S.
8. Wink M. *Encyclopedia of Plant and Crop Science*. Marcel Dekker, 2004.
9. Chou T. Wake up and smell the coffee. Caffeine, coffee, and the medical consequences. *West J Med* 1992;157:544-53.
10. Dinkova-Kostova AT. Phytochemicals as protectors against ultraviolet radiation: versatility of effects and mechanisms. *Planta Med* 2008;74:1548-59.
11. Juge N, Mithen RF, Traka M. Molecular basis for chemoprevention by sulforaphane: a comprehensive review. *Cell Mol Life Sci* 2007;64:1105-27.
12. Stahl W, Sies H. Antioxidant activity of carotenoids. *Mol Aspects Med* 2003;24:345-51.
13. Rao AV, Rao LG. Carotenoids and human health. *Pharmacol Res* 2007;55:207-16.
14. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 2004;79:727-47.
15. Slimestad R, Fossen T, Vagen IM. Onions: a source of unique dietary flavonoids. *J Agric Food Chem* 2007;55:10067-80.
16. Somerset SM, Johannot L. Dietary flavonoid sources in Australian adults. *Nutr Cancer* 2008;60:442-9.
17. Shan B, Cai YZ, Sun M, Corke H. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *J Agric Food Chem* 2005;53:7749-59.
18. Holst B, Williamson G. Nutrients and phytochemicals: from bioavailability to bioefficacy beyond antioxidants. *Curr Opin Biotechnol* 2008;19:73-82.

-
19. van het Hof KH, de Boer BC, Tijburg LB et al. Carotenoid bioavailability in humans from tomatoes processed in different ways determined from the carotenoid response in the triglyceride-rich lipoprotein fraction of plasma after a single consumption and in plasma after four days of consumption. *J Nutr* 2000;130:1189-96.
 20. Gilca M, Stoian I, Atanasiu V, Virgolici B. The oxidative hypothesis of senescence. *J Postgrad Med* 2007;53:207-13.
 21. Bhattacharyya S, Dudeja PK, Tobacman JK. Lipopolysaccharide activates NF-kappaB by TLR4-Bcl10-dependent and independent pathways in colonic epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 2008;295:G784-G790.
 22. Blomhoff R. Antioksidanter - Den sanne historien. Kagge Forlag, 2008.
 23. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007;39:44-84.
 24. Valen G, Yan ZQ, Hansson GK. Nuclear factor kappa-B and the heart. *J Am Coll Cardiol* 2001;38:307-14.
 25. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* 2006;160:1-40.
 26. Muller FL, Lustgarten MS, Jang Y, Richardson A, Van RH. Trends in oxidative aging theories. *Free Radic Biol Med* 2007;43:477-503.
 27. Bjelakovic G, Nikolova D, Simonetti RG, Gluud C. Antioxidant supplements for prevention of gastrointestinal cancers: a systematic review and meta-analysis. *Lancet* 2004;364:1219-28.
 28. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. *Cochrane Database Syst Rev* 2008;CD007176.
 29. Miller ER, III, Guallar E. Vitamin E supplementation: what's the harm in that? *Clin Trials* 2009;6:47-9.
 30. Virgili F, Marino M. Regulation of cellular signals from nutritional molecules: a specific role for phytochemicals, beyond antioxidant activity. *Free Radic Biol Med* 2008;45:1205-16.
 31. Khan N, Afaq F, Mukhtar H. Cancer chemoprevention through dietary antioxidants: progress and promise. *Antioxid Redox Signal* 2008;10:475-510.
 32. Son TG, Camandola S, Mattson MP. Hormetic dietary phytochemicals. *Neuromolecular Med* 2008;10:236-46.
 33. Gilmore TD, Herscovitch M. Inhibitors of NF-kappaB signaling: 785 and counting. *Oncogene* 2006;25:6887-99.
 34. Paur I, Austenaa LM, Blomhoff R. Extracts of dietary plants are efficient modulators of nuclear factor kappa B. *Food Chem Toxicol* 2008;46:1288-97.
 35. Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2002;2:301-10.

-
36. Naugler WE, Karin M. NF-kappaB and cancer-identifying targets and mechanisms. *Curr Opin Genet Dev* 2008;18:19-26.
 37. Aggarwal BB, Shishodia S. Suppression of the nuclear factor-kappaB activation pathway by spice-derived phytochemicals: reasoning for seasoning. *Ann N Y Acad Sci* 2004;1030:434-41.
 38. Gabrielsen, Odd Stokke. The NF- κ B/Rel family. 2008. The NF- κ B/Rel family. Ref Type: Slide
 39. Karin M, Yamamoto Y, Wang QM. The IKK NF-kappa B system: a treasure trove for drug development. *Nat Rev Drug Discov* 2004;3:17-26.
 40. Carlsen H, Alexander G, Austenaa LM, Ebihara K, Blomhoff R. Molecular imaging of the transcription factor NF-kappaB, a primary regulator of stress response. *Mutat Res* 2004;551:199-211.
 41. Gilmore Thomas D. The Rel/NF-kappaB Signal Transduction Pathway. 6-3-2009. Ref Type: Internet Communication
 42. Viatour P, Merville MP, Bours V, Chariot A. Phosphorylation of NF-kappaB and IkkappaB proteins: implications in cancer and inflammation. *Trends Biochem Sci* 2005;30:43-52.
 43. Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 1999;18:6853-66.
 44. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57-70.
 45. Perkins ND. The Rel/NF-kappa B family: friend and foe. *Trends Biochem Sci* 2000;25:434-40.
 46. Epinat JC, Gilmore TD. Diverse agents act at multiple levels to inhibit the Rel/NF-kappaB signal transduction pathway. *Oncogene* 1999;18:6896-909.
 47. Paur, I, Balstad, T. R, and Blomhoff, R. Degree of roasting is the main determinant of the effects of coffee on NF- κ B activity and EpRE-dependent transcription. 2009. Ref Type: Personal Communication
 48. Keith V.Wood. The Chemistry of Bioluminescent Reporter Assays. *Promega Notes* (65). 1998. Ref Type: Generic
 49. Berger F, Paulmurugan R, Bhaumik S, Gambhir SS. Uptake kinetics and biodistribution of (14)C-D: -luciferin-a radiolabeled substrate for the firefly luciferase catalyzed bioluminescence reaction: impact on bioluminescence based reporter gene imaging. *Eur J Nucl Med Mol Imaging* 2008;35:2275-85.
 50. Carlsen H, Moskaug JO, Fromm SH, Blomhoff R. In vivo imaging of NF-kappa B activity. *J Immunol* 2002;168:1441-6.
 51. Kile BT, Hilton DJ. The art and design of genetic screens: mouse. *Nat Rev Genet* 2005;6:557-67.
 52. Clamp M, Fry B, Kamal M et al. Distinguishing protein-coding and noncoding genes in the human genome. *Proc Natl Acad Sci U S A* 2007;104:19428-33.

-
53. Southan C. Has the yo-yo stopped? An assessment of human protein-coding gene number. *Proteomics* 2004;4:1712-26.
54. Peters LL, Robledo RF, Bult CJ, Churchill GA, Paigen BJ, Svenson KL. The mouse as a model for human biology: a resource guide for complex trait analysis. *Nat Rev Genet* 2007;8:58-69.
55. Moores Cancer Centre.San Diego Medical Centre.Univerity of California . A Practical Guide for Making Transgenic Mice. 1-3-2009.
Ref Type: Generic
56. Kolberg, M. Additive and synergistic modulations of nuclear factor kB by dietary plant extracts. 2008. Department of nutrition, faculty of medicine, University of Oslo.
Ref Type: Thesis/Dissertation
57. Carlsen, H. Diet and gene regulation: Molecular imaging of gene expression in transgenic reporter mice. 2003. Institute for nutrition research. Institute of basic medical sciences. Faculty of medicine. University of Oslo.
Ref Type: Thesis/Dissertation
58. Corral-Aguayo RD, Yahia EM, Carrillo-Lopez A, Gonzalez-Aguilar G. Correlation between some nutritional components and the total antioxidant capacity measured with six different assays in eight horticultural crops. *J Agric Food Chem* 2008;56:10498-504.
59. Halvorsen BL, Holte K, Myhrstad MC et al. A systematic screening of total antioxidants in dietary plants. *J Nutr* 2002;132:461-71.
60. Renuka DR, Arumughan C. Phytochemical characterization of defatted rice bran and optimization of a process for their extraction and enrichment. *Bioresour Technol* 2007;98:3037-43.
61. Dragland S, Senoo H, Wake K, Holte K, Blomhoff R. Several culinary and medicinal herbs are important sources of dietary antioxidants. *J Nutr* 2003;133:1286-90.
62. Cai Y, Luo Q, Sun M, Corke H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci* 2004;74:2157-84.
63. Zheng W, Wang SY. Antioxidant activity and phenolic compounds in selected herbs. *J Agric Food Chem* 2001;49:5165-70.
64. Paur, I. Balstad T R. Kolberg M. Pedersen M K. Jacobs D R Jr. Austenaa L M. Blomhoff R. Extract of oregano, coffee, thyme, clove and walnuts inhibits Nuclear Factor kappa B in monocytes and in transgenic reporter mice. 2009.
Ref Type: Unpublished Work
65. Abraham SK, Vukicevic V, Stopper H. Coffee-mediated protective effects against directly acting genotoxins and gamma-radiation in mouse lymphoma cells. *Cell Biol Toxicol* 2004;20:121-32.
66. Fujioka K, Shibamoto T. Quantitation of volatiles and nonvolatile acids in an extract from coffee beverages: correlation with antioxidant activity. *J Agric Food Chem* 2006;54:6054-8.
67. Shimoda H, Tanaka J, Kikuchi M et al. Walnut polyphenols prevent liver damage induced by carbon tetrachloride and d-galactosamine: hepatoprotective hydrolyzable tannins in the kernel pellicles of walnut. *J Agric Food Chem* 2008;56:4444-9.

-
68. Colaric M, Veberic R, Solar A, Hudina M, Stampar F. Phenolic acids, syringaldehyde, and juglone in fruits of different cultivars of *Juglans regia* L. *J Agric Food Chem* 2005;53:6390-6.
69. Ros E. Nuts and novel biomarkers of cardiovascular disease. *Am J Clin Nutr* 2009;89:1649S-56S.
70. Li N, Karin M. Is NF-kappaB the sensor of oxidative stress? *FASEB J* 1999;13:1137-43.
71. Vitrac X, Desmouliere A, Brouillaud B et al. Distribution of [14C]-trans-resveratrol, a cancer chemopreventive polyphenol, in mouse tissues after oral administration. *Life Sci* 2003;72:2219-33.
72. Suganuma M, Okabe S, Oniyama M, Tada Y, Ito H, Fujiki H. Wide distribution of [3H](-)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. *Carcinogenesis* 1998;19:1771-6.
73. Mullen W, Graf BA, Caldwell ST et al. Determination of flavonol metabolites in plasma and tissues of rats by HPLC-radiocounting and tandem mass spectrometry following oral ingestion of [2-(14C)]quercetin-4'-glucoside. *J Agric Food Chem* 2002;50:6902-9.
74. Bobe G, Wang B, Seeram NP, Nair MG, Bourquin LD. Dietary anthocyanin-rich tart cherry extract inhibits intestinal tumorigenesis in APC(Min) mice fed suboptimal levels of sulindac. *J Agric Food Chem* 2006;54:9322-8.
75. Kim YH, Kwon HS, Kim DH et al. 3,3'-diindolylmethane attenuates colonic inflammation and tumorigenesis in mice. *Inflamm Bowel Dis* 2009.
76. Botsoglou NA, Taitzoglou IA, Botsoglou E, Lavrentiadou SN, Kokoli AN, Roubies N. Effect of long-term dietary administration of oregano on the alleviation of carbon tetrachloride-induced oxidative stress in rats. *J Agric Food Chem* 2008;56:6287-93.
77. Sasaki K, Wada K, Tanaka Y, Yoshimura T, Matuoka K, Anno T. Thyme (*Thymus vulgaris* L.) leaves and its constituents increase the activities of xenobiotic-metabolizing enzymes in mouse liver. *J Med Food* 2005;8:184-9.
78. Huber WW, Scharf G, Rossmanith W et al. The coffee components kahweol and cafestol induce gamma-glutamylcysteine synthetase, the rate limiting enzyme of chemoprotective glutathione synthesis, in several organs of the rat. *Arch Toxicol* 2002;75:685-94.
79. Reddy DB, Reddanna P. Chebulagic acid (CA) attenuates LPS-induced inflammation by suppressing NF-kappaB and MAPK activation in RAW 264.7 macrophages. *Biochem Biophys Res Commun* 2009;381:112-7.
80. Li W, Khor TO, Xu C et al. Activation of Nrf2-antioxidant signaling attenuates NFkappaB-inflammatory response and elicits apoptosis. *Biochem Pharmacol* 2008;76:1485-9.
81. Liu GH, Qu J, Shen X. NF-kappaB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochim Biophys Acta* 2008;1783:713-27.
82. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. *N Engl J Med* 1994;330:1029-35.

-
83. Omenn GS, Goodman GE, Thornquist MD et al. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N Engl J Med* 1996;334:1150-5.

9. Appendix

1. Results from the combination extract experiment performed within this thesis.
2. Paur,I. Balstad,T R. Kolberg,M. Pedersen M K. Jacobs,D R Jr. Austenaa,L M. Blomhoff,R. **Extract of oregano, coffee, thyme, clove and walnuts inhibits Nuclear Factor kappa B in monocytes and in transgenic reporter mice.**
Unpublished work.

Appendix 1:

Results from the combination extract experiment performed within this thesis.

Effect of combination extract on LPS-induced NF- κ B activity

Transgenic NF- κ B-luciferase reporter mice were given combination extract per os in a single dose 3 h prior to LPS injection (0h). NF- κ B activation was measured by *in vivo* imaging at -3, 0, 2, 4, 6h, *ex vivo* imaging (at 6h) and LUC assay of individual organs. Sample size: n=4 control, n=4 extract.

In vivo imaging

Quantification of light emitted from whole mice revealed a trend towards inhibition of NF- κ B activation in the mice receiving the combination extract compared to control mice. One representative mouse from each treatment is shown in **figure 9.1A**.

At 2 hours after LPS injection, activation in the extract group was 37 % lower than in the control group (**Figure 9.1B**). At 6 h, it was 25 % lower. Total area under curve (AUC) (0-6 h) is 48.19 (95% CI: 18.11-78.27) for the extract group and 80.65 (95% CI: 33.80-127.48) for the control group (**Figure 9.1C**). This represents a 0.6 fold lower activation in the combination extract group compared to the control group.

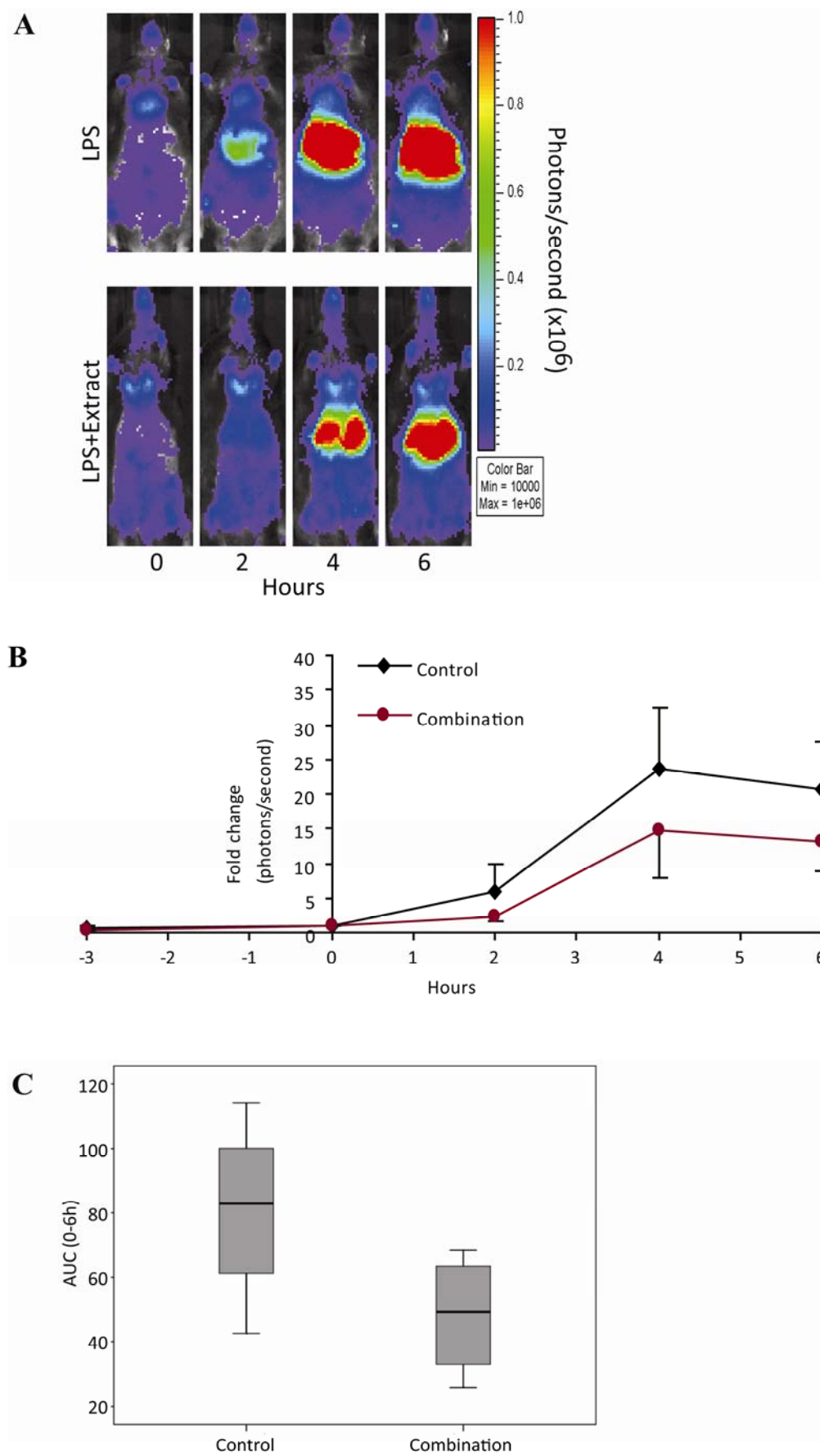


Figure 9.1. Effect of combination extract in transgenic NF- κ B reporter mice. *In vivo* imaging. Mice were given combination extract or control vehicle 3 h prior to LPS injection (0h). A) *In vivo* images of one representative mouse from each group. B) Mean \pm SD fold change of luminescence (photons/second) from the whole mouse minus head and extremities. C) Box plot of area under curve (AUC) for control group and combination extract group.

Ex vivo imaging

Organ specific NF- κ B activation can not be detected by *in vivo* imaging. To evaluate differences organs (brain, lung, heart, thymus, spleen, kidney, liver, intestines, skin, muscle, adipose tissue, testicle and epididymis) were excised and NF- κ B activation was measured by *ex vivo* imaging.

Ex vivo imaging after administration of the combination extract or vehicle control revealed that the combination extract was able to inhibit NF- κ B activation in the epididymis (**Figure 9.2B**). The mean fold change is 0.62 ± 0.07 . No other organs showed significant differences, although there seems to be a lower NF- κ B activation in intestine and liver, and possibly an increased NF- κ B activation in spleen, lung and skin.

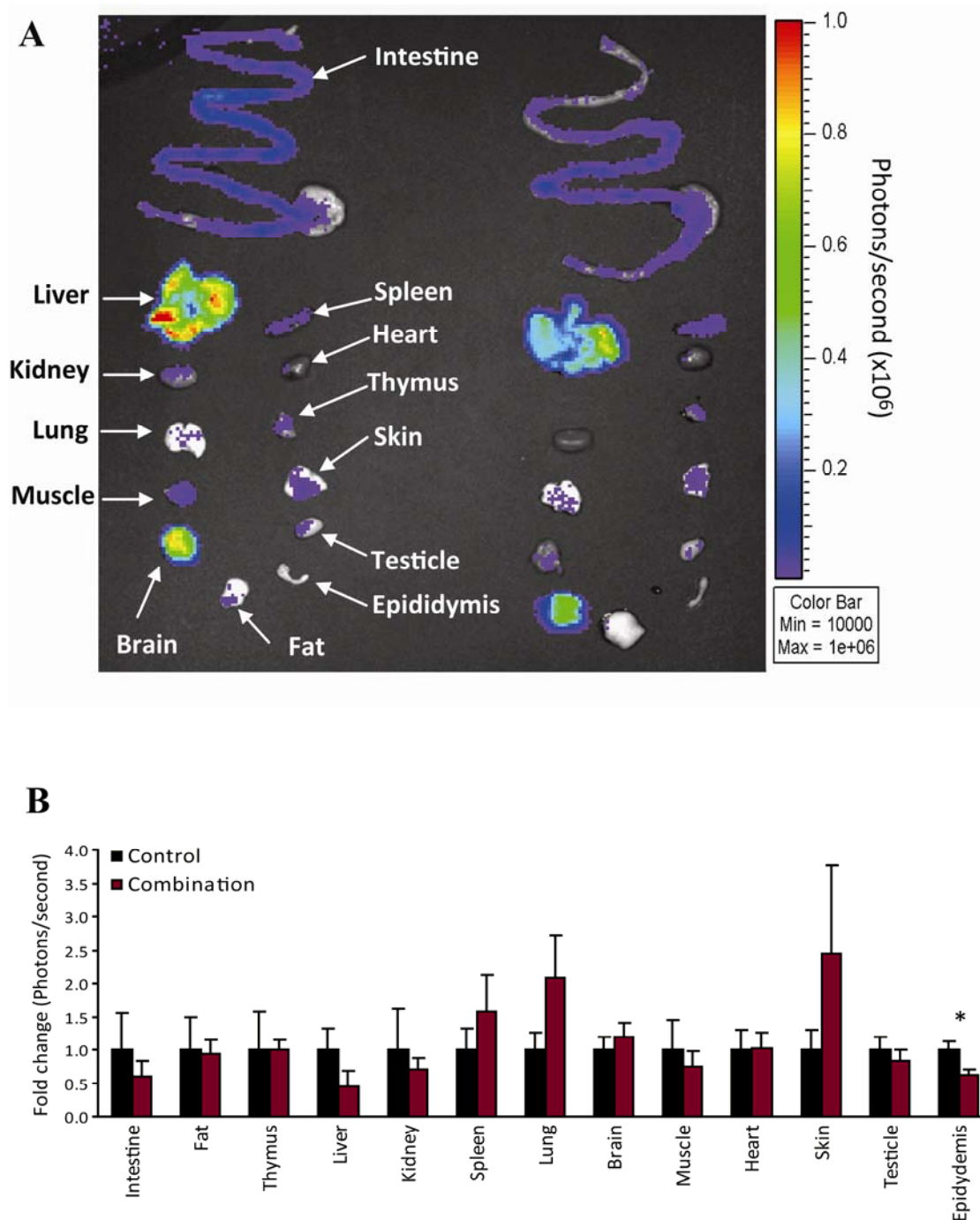


Figure 9.2. Effect of combination extract in transgenic NF- κ B reporter mice. *Ex vivo* imaging.

LPS induced luciferase activity in organs after administration of combination extract or control vehicle. Organs were excised and imaged 6 h post LPS injection, luminescence was measured by imaging in an IVIS 100 imaging system, and quantified using the “Living Image software”. Gray scale images were used for reference of position. A: Representative organs from one control mouse (left) and one combination extract mouse (right). The orders of the organs are identical for both sets. B: Quantification of emitted light from organs. Bars represent mean fold change + SEM. * = $p < 0.05$

LUC assay

NF- κ B activity was measured in homogenates of organs (brain, lung, heart, thymus, spleen, kidney, liver, muscle, adipose tissue, testicle and epididymis).

The NF- κ B activity (**Figure 9.3**) decreased in testicle and epididymis in the combination extract group compared to the control group. The mean fold change was 0.75 ± 0.04 , and 0.58 ± 0.02 , respectively. There might be a trend towards increased NF- κ B activity in the spleen.

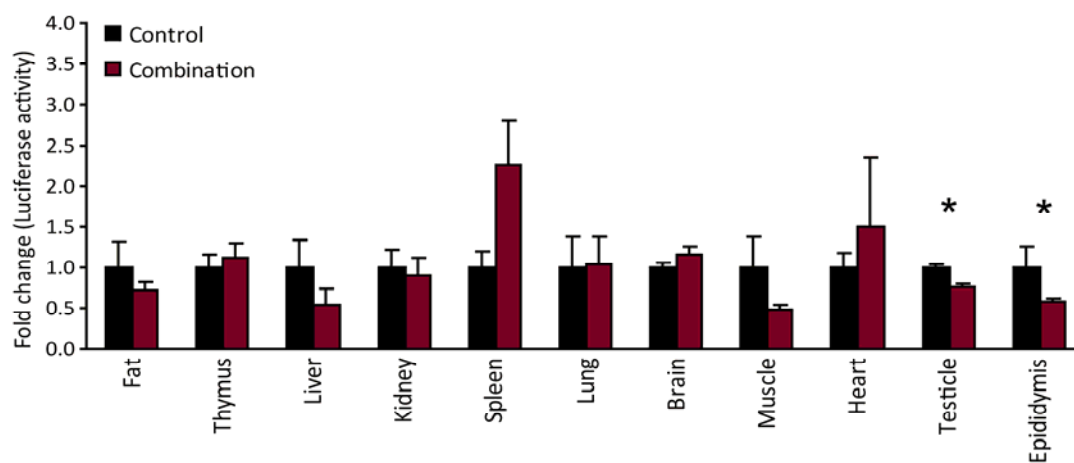


Figure 9.3. Effect of combination extract in transgenic NF- κ B reporter mice. LUC assay.

LPS induced luciferase activity in organs after administration of combination extract or control vehicle. Organs were excised 6 h post LPS injection, measured for luciferase activity and corrected for total protein content. Bars represent mean fold change + SEM. * = $p < 0.05$

Appendix 2:

Paur,I. Balstad,T R. Kolberg,M. Pedersen M K. Jacobs,D R Jr. Austenaa,L M.

Blomhoff,R. **Extract of oregano, coffee, thyme, clove and walnuts inhibits Nuclear Factor kappa B in monocytes and in transgenic reporter mice.**

Unpublished work.

Extract of oregano, coffee, thyme, clove and walnuts inhibits nuclear factor kappa B in monocytes and in transgenic reporter mice

Ingvild Paur¹, Trude R. Balstad¹, Marit Kolberg¹, Marit K. Pedersen¹, Liv M. Austenaa², David R. Jacobs Jr.^{1,3}, and Rune Blomhoff¹

¹ Department of Nutrition, Faculty of Medicine, University of Oslo, Norway. ² The FIRC Institute of Molecular Oncology Foundation, European Institute of Oncology, Milan, Italy. ³ Division of Epidemiology and Community Health, School of Public Health, University of Minnesota, Minnesota, USA.

Requests for reprints: Rune Blomhoff, Department of Nutrition, Faculty of Medicine, University of Oslo, P.O. Box 1046, 0316 Oslo, Norway, Fax: + 47 22851396; E-mail: rune.blomhoff@medisin.uio.no.

Running Title: Clove, oregano, thyme, walnuts and coffee inhibit NF-κB *in vivo*

Keywords: NF-κB, diet, synergy, transgenic mice, *in vivo* imaging

Financial support: This work was supported by grants from the Norwegian Cancer Society (IP, LMA and RB) and the Johan Throne Holst Foundation (RB and DRJ).

Acknowledgements: The U937-κB cells were generously donated by Christian Drevon, Dept. of Nutrition, University of Oslo. We thank Hanne Reiersen, Lene Gilen and Kari Holte for technical assistance and Cgene for providing the IVIS100 imaging system and the reporter mice.

Conflicts of interest: RB has owner interests in Cgene AS which was established by Birkeland Innovation (the Technology transfer office at the University of Oslo) and holds the commercial rights to the transgenic NF-κB-luciferase reporter mice. DRJ is an unpaid member of the Scientific Advisory Council of the California Walnut Commission.

Abbreviations used: Nuclear Factor kappa B: NF-κB, Lipopolysaccharide: LPS, Inhibitor of NF-κB: IκB, IκB Kinase: IKK, Interleukin: IL, Tumor Necrosis Factor: TNF, Dimethylsulfoxide: DMSO, Ferric Reducing Ability of Plasma: FRAP, subcutaneously: s.c.

Abstract

The transcription factor nuclear factor kappa B (NF-κB) is a promising target for chemoprevention. Several dietary plants are efficient inhibitors of NF-κB activation *in vitro* and could act synergistically on the NF-κB signaling pathway. In this study, we tested whether dietary plant extracts could inhibit NF-κB activation in a synergistic manner *in vitro*. Secondly, we investigated the potency of the same dietary plant extracts in inhibition of NF-κB activation *in vivo*. A combined extract of clove, oregano, thyme, walnuts

and coffee synergistically inhibited lipopolysaccharide (LPS)-induced NF-κB activation in a monocytic cell line, as compared to the sum of effects from the single extracts. Transgenic NF-κB-luciferase reporter mice were administered a single dose of the combined extract, and subsequently challenged with LPS. NF-κB activation was monitored by *in vivo* imaging for 6h. Also, organ specific inhibition of NF-κB was investigated. Based on area under the curve, the extract decreased whole body LPS-induced NF-κB activity the first 6 hours by 35 % as compared to control mice. Organ specific NF-κB activation was inhibited in intestine and liver (to 53 % and 67 % of controls, respectively), and as well as in testis and epididymis (to 66 % and 48 %, respectively) of the mice receiving the combination extract. This study shows that dietary plants may be potent modulators of the NF-κB signaling pathway both *in vitro* and *in vivo*, and thus support further investigation of consumption of these plant foods as part of a healthy diet or as a mode of chemoprevention.

Introduction

Recent studies have identified nuclear factor kappa B (NF-κB) as a direct link between inflammation and cancer (1-3) and thus renders this family of transcription factors as a key molecular target for both prevention and treatments of cancers. The NF-κB family of transcription factors are crucial mediators of cellular stress-, immune- and inflammatory responses (4). Although NF-κB is essential in normal physiology, several human disorders involve inappropriate regulation of NF-κB. Several chronic degenerative diseases (5-9) and various human cancers (10) have been associated with an aberrant up-regulation of NF-κB activity. Both the NF-κB activation and cytokine profile of tumor-associated macrophages are closely linked to tumor growth (11;12).

In vertebrates, the NF-κB family is comprised of five structurally related proteins (p65 (REL A), p50, p52, c-REL, REL B) which form hetero- and homodimers. Normally the NF-κB dimers are sequestered in the cytoplasm by binding to inhibitory factors. Two pathways lead to delocalization of NF-κB dimers to the nucleus and consequent transcriptional regulation of target genes (13). The classical NF-κB signaling pathway is activated by pro-inflammatory stimuli, bacterial- or viral infection, and various forms of stress

such as UV-radiation and environmental toxins (14), and is essential for the innate immune system and anti-apoptotic signaling. The alternative NF- κ B pathway plays a critical role for both development and maintenance of the adaptive immune system, and is activated by members of the TNF family other than TNF- α (13). NF- κ B target genes code for proteins which are central players in inflammation, activation of the immune system and anti-apoptotic signaling, which can be involved in both promotion and progression of cancers (15). With the central role of NF- κ B activation in inflammation, cancer and anti-apoptosis, substances with the ability to inhibit NF- κ B activation may be valuable in a generally healthy diet, in chemoprevention (16) and in combination with chemotherapy (17).

Plants and plant-based diets contain thousands of phytochemicals (18) in addition to well known macro- and micronutrients. In epidemiological studies, diets rich in plant-based foods reduce the risk of several cancers (19), and several chronic diseases (20). Clinical trials with single compounds have, however, not been able to reproduce these risk reductions (19;21;22). For these reasons, it is likely that several of these compounds work in additive, synergistic or antagonistic fashions to create the observed health effects, and thus foods or combinations of foods may be better suited as the basic unit for research on these preventive effects (23). The preventive properties of such diets are likely to be complex combinations of numerous mechanisms. None the less, aberrant cellular signaling is a hallmark of carcinogenesis, and targeting crucial signaling pathways, such as the NF- κ B pathway, yield valuable progress towards elucidation of the mechanisms underlying chemoprevention. We previously screened a wide range of dietary plants and phytochemicals for their effect on NF- κ B activation and found clove, thyme, oregano, coffee and walnuts to be the most potent inhibitors (24). In this study, we demonstrate that an extract of these five dietary plants synergistically inhibit NF- κ B activation *in vitro*, and can also inhibit NF- κ B activation *in vivo* in transgenic mice. Interestingly, lowered NF- κ B activation was detected in the liver and intestine, as well as in testis and epididymis of male mice, indicating organ specific effects of the dietary plants.

Materials and Methods

Cell culture

U937- κ B cells are previously described (25). Cells were cultured in RPMI-1640 medium with L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 mg/ml), hygromycin (75 μ g/ml), and 10% fetal bovine serum at 37°C and 5 % CO₂. During experiments, cells were seeded in medium with 2% fetal bovine serum. NF- κ B activity was induced by

lipopolysaccharide (LPS) (1 μ g/ml). Cell viability was determined by trypan blue exclusion. A cut-off value of 10 % non-viable cells was used.

Dietary plant extract

Coffee (Arabica, Medium roast), walnuts, oregano (dried), clove (dried), and thyme (dried) were purchased from a grocery store in Oslo, Norway. All samples were pulverized and 10g coffee, 10g walnuts plus 1g of each of oregano, thyme and clove were added equal volumes of water and methanol. The mixture was placed in an ultrasonic water bath for 30 min at 0°C. The mixture was centrifuged at 3000 g for 15 min, the liquid phase saved. Centrifugation was repeated to increase the yield of the liquid phase, thereafter the total liquid phase was concentrated under nitrogen gas to a viscid fluid. The concentrated extract was diluted to 5 ml in corn oil (extract used for mice) or a 50:50 mix of phosphate buffered saline (PBS):dimethylsulfoxide (DMSO) (v/v). The extract to be used for cell culture was thereafter sterile filtered and stored under argon gas in airtight tubes at -70°C.

Transgenic mice

The mice were housed in accordance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) and animal experiments were performed according to national guidelines for animal welfare. Transgenic mice with B6 background carrying a transgene with three binding sites for NF- κ B coupled to the luciferase gene have previously been described (25). A single dose of an extract corresponding to 60 mg each of thyme, oregano and clove, and 600 mg each of walnuts and coffee or vehicle control (corn oil), was administered by oral gavage to the mice (both groups: n = 16, 10 female and 6 male, 11-24 weeks of age). No differences were found between the female and male mice, and thus the results from both sexes were pooled. LPS (2.5 mg/kg) in 100 μ l PBS was injected subcutaneously (s.c.) on the back near the tail 3h after oral gavage. *In vivo* imaging was performed at the given time points. Directly following imaging at 6h, the mice were euthanized, *ex vivo* imaging of the intestine was performed, and all other organs were frozen in liquid nitrogen for further analysis. Luciferase activity in homogenates of organs was measured as previously described (25).

Luciferase activity

Luciferase activity was measured by use of an IVIS 100 Imaging System (Xenogen Corp., USA). D-luciferin (0.2 mg/ml medium) was added to the cell culture medium, and cells were incubated at 37°C for 4 min. Mice were anesthetized using 2.5 % isoflurane, and were shaved on the abdomen. D-luciferin (Biosynth AG, Switzerland) (160 mg/kg) in PBS was injected intraperitoneally, and mice were imaged 7 minutes after injection of D-luciferin. Cell culture plates or mice were placed in a light proof chamber, and the

luminescence emitted from the cells/mice was detected for 1 min. *Ex vivo* imaging of the intestine was performed 20 min after the injection of D-luciferin. The number of photons emitted was calculated by use of the Living Image Software (Xenogen Corp., USA). Gray scale images were used for reference of position.

Statistical analysis for *in vitro* experiments

The U937- κ B cell cultures may vary in their NF- κ B response to LPS depending on the passage of the cells. Therefore the control (no extract) response to LPS was idiosyncratic for each experiment, and the most stable set of experimental conditions is within a particular experiment. For these reasons, we set the control LPS level as 1, meaning 100% of NF- κ B activity, and computed the responses to the LPS plus extract treatments as fold changes compared to controls.

One-way ANOVA was used to examine effects of extracts, on NF- κ B activity in U937- κ B cells. Differences were identified using Dunnett's comparisons.

For the calculations of synergy, we used the natural logarithm of the NF- κ B activity as the response metric, which takes a value of 0 for the control situation (response to LPS alone), a negative number for inhibition of the NF- κ B activity to LPS plus extract, and a positive number for accentuation of the activity. We ran a linear regression model with dependent variable $\ln(\text{NF-}\kappa\text{B})$, with no intercept, that included as independent variables all 5 main effects of extracts, and a single term representing the combined extract. As the main test of synergy, we then estimated the contrast for the combined extract effect minus the sum of the 5 corresponding main effects.

Statistical analysis for *in vivo* experiments

For *in vivo* experiments the area under the curve (AUC) of LPS-induced luciferase activity from 0 hours to 6 hours, was calculated for each mouse. Student's t-tests were performed to compare the data from AUC, at each time point, from *ex vivo* imaging, and from luciferase activity in tissue homogenates. Statistically significant difference was set to $p < 0.05$ for all analysis. Results are presented as mean \pm SEM, unless otherwise noted.

Results

Combined extract inhibits NF- κ B activation in U937- κ B cells

An extract made from the combination of clove, oregano, thyme, walnuts and coffee inhibited LPS-induced NF- κ B activity in the U937- κ B cells in a dose dependent manner (Fig. 1A). In the U937- κ B cells,

basal NF- κ B activity was 0.05 ± 0.009 fold (5%) of LPS-induced NF- κ B activity (Fig. 1A). The extract made from 0.3 mg/ml coffee- and walnut-, and 0.03 mg/ml thyme-, oregano- and clove reduced LPS-induced NF- κ B activation to 0.74 ± 0.05 fold ($p < 0.001$) of LPS-controls, while the extract made of 0.6 mg/ml coffee and walnut, and 0.06 mg/ml thyme, oregano and clove further reduced luciferase activity to 0.51 ± 0.04 fold ($p < 0.001$) of LPS-controls. Increasing the concentration to 1.5/0.15 mg/ml and 3/0.3 mg/ml (coffee and walnut/thyme, oregano, clove) almost completely blocked LPS-induced NF- κ B activation to respectively 0.09 ± 0.02 fold and 0.08 ± 0.01 fold ($p < 0.001$ for both) without cytotoxicity.

Assessment of Synergy

To test for synergy, we calculated the difference between the effects of the combined extract and the sum of effects for the 5 single ingredients tested separately. Effects of single ingredients are shown in Fig. 1B. When comparing equal concentrations of the ingredients (3 mg/ml each of coffee and walnut, and 0.3 mg/ml each of thyme, oregano and clove), the combined extract inhibited NF- κ B significantly more than the expected additive inhibitory effects from the 5 ingredients tested separately (Table 1A) indicating synergistic effects by the combination extract. At combined extract concentration of 1.5 mg/ml each of coffee and walnut, and 0.15 mg/ml each of thyme, oregano and clove, the combined extract was still significantly more potent in inhibiting NF- κ B activation as compared to the expected sum of inhibitions from the 5 ingredients at 3 mg/ml coffee and walnut, and 0.3 mg/ml thyme, oregano and clove (Table 1B). The combined extract concentration of 0.6 mg/ml of coffee and walnut and 0.06 mg/ml of thyme, oregano and clove, was equally potent as the expected sum of the effects for 3 mg/ml of coffee and walnut, and 0.3 mg/ml of thyme, oregano and clove (Table 1C). A way of expressing the synergy is to note that 1/5th the total volume of food extract was needed to obtain equal NF- κ B inhibition using the combined extract as was expected based on the separate effects of the individual food extracts.

The combination extract inhibits NF- κ B in transgenic mice

Next, we investigated whether the combination extract could modulate LPS-induced NF- κ B activity *in vivo*. An s.c. injection of LPS (160 mg/kg) increased NF- κ B activity to a maximum of 15.7 ± 2.4 fold ($n = 16$) after 4 hours as measured by whole body *in vivo* imaging of the luciferase activity. The extract or control vehicle (corn oil) was administered by oral gavage to mice 3 hours (at time -3 h) prior to s.c. LPS-injection (at time 0h). Whole body *in vivo* imaging was performed at 0, 2, 4 and 6 hours (Fig 2A & B). At 2h ($p = 0.043$) and 6h ($p = 0.041$) after LPS-injection, the extract significantly inhibited the LPS-induced NF- κ B activity, compared to mice treated with LPS only. Based on the AUC, the

extract decreased LPS-induced NF- κ B activity the first 6 hours by 35 % ($p = 0.040$) as compared to control mice (Fig. 2C).

Following *in vivo* imaging at 6h, the mice were euthanized, and luminescence was measured in intestine by *ex vivo* imaging and in homogenates of other tissues. There was a significant reduction in NF- κ B activity to 0.53 fold ($p = 0.028$) of controls, in the intestine of mice receiving LPS plus the extract as compared to mice receiving LPS only, as measured by *ex vivo* imaging (Fig. 3A). Representative intestines from the LPS-only and the LPS + extract treated mice are shown in Fig. 3B. Furthermore, comparing luciferase activity by luminometry of tissue homogenates of organs from mice receiving the extract to mice receiving LPS-only, revealed significantly decreased NF- κ B activity in the liver (0.67 fold of control, $p = 0.046$), testis (0.66 fold of controls, $p = 0.007$) and epididymis (0.48 fold of control, $p = 0.021$) (Fig. 4C). In the spleen the NF- κ B activity was further increased in the mice receiving the extract (1.57 fold of control, $p = 0.047$). Although there was a suggestive but not significant increase in heart NF- κ B activity, no other differences in luciferase activity were found in brain, thymus, lung, kidney, fat, muscle, or uterus.

Discussion

Inhibitors of NF- κ B carry great promise for both prevention and therapy in cancers and chronic inflammation. An extract made from coffee, walnut, thyme, oregano and clove inhibited NF- κ B activation in U937- κ B cells in a synergistic fashion as compared to the effects of the single extracts. Furthermore, by using non-invasive molecular imaging of transgenic NF- κ B luciferase reporter mice we demonstrated that the same combined extract efficiently inhibit LPS-induced NF- κ B activation *in vivo*.

As recently demonstrated NF- κ B is a direct link between inflammation and cancer (1-3) and aberrant activation of NF- κ B has been found in several types of human cancers (10). Inflammation coordinated through NF- κ B activation will most often promote tumor growth (15), and inflammation-promoted tumor growth can be turned to tumor regression by inhibition of NF- κ B in cancerous cells (2). NF- κ B is essential for survival and cytokine production of monocytes and macrophages (26), and further NF- κ B activation in macrophages is associated with cancer development. The cytokine profiles of monocytes and macrophages are also closely linked to tumor growth (11;12). The observed decrease in LPS-induced NF- κ B activation in the monocytic cell line as well as macrophage rich organs *in vivo* (i.e., liver and intestine) following administration of plant extracts suggest that such plant extracts may have chemopreventive effects. NF- κ B inhibitors therefore have great potential both in cancer

prevention and as parts of a chemotherapeutic regime (17). Also, to the extent that inflammation is an important factor in disease conditions other than cancer, the consumption of these foods should be regarded as part of a generally healthy diet.

The inhibition of NF- κ B activity in liver, testis and epididymis indicate that compounds from the extract have been absorbed and are bioactive after absorption. In the intestine, the extract may produce its effect both by acting in the intestinal lumen and after absorption into intestinal cells. Further work should be done to identify the bioactive compounds as well as the mechanisms of action, in all four organs. The intestine and liver are the main organs for metabolism of phytochemicals. There is limited knowledge on tissue distribution of phytochemicals present in our extract. However, quercetin, resveratrol and epigallocatechin gallate (EGCG) have, however, previously been detected in the intestine, liver, and testes of mice and rats (27-29). Consistent with these results, we would expect the highest concentrations of phytochemicals from our extract in these organs, and thus also a greater chance of detectable effects on a major signaling pathway such as NF- κ B.

Induction of another transcription factor; nuclear factor erythroid 2-related factor 2 (Nrf2), may affect the organ specificity of NF- κ B responses as there are lines of evidence suggesting a cross-talk between the NF- κ B and Nrf2 signaling pathways (30-32), and this cross-talk is most likely to be evident in organs with the highest Nrf2-activity. A wide range of phytochemicals are known to activate Nrf2 leading to induced expression of phase 2 enzymes and endogenous antioxidants (collectively called cytoprotective proteins (33)), and many of these phytochemicals are also known to inhibit NF- κ B (32). Like NF- κ B, Nrf2 has been implicated in carcinogenesis and inflammation both in liver and the gastrointestinal tract (34-36), and an induction of cytoprotective proteins is an alternative approach to reducing the tissue damage caused by carcinogens or excessive inflammatory response. Phytochemicals are mainly metabolized in the intestine and liver. Thus, other organs will be exposed to metabolites or conjugates of the ingested compounds, possibly resulting in organ specific NF- κ B responses. In the spleen and possibly in the heart, the LPS-induced NF- κ B activation was further increased in the mice receiving extract. It should be noted, however, that the absolute NF- κ B activity (adjusted for protein contents of the samples) in the liver is 8 fold and 4 fold higher than in spleen and heart respectively. In combination with the large size of the liver, this implies that the liver is contributing considerably more than any other organs to the overall NF- κ B activity in the mice. We speculate that it would be an advantage to maintain a high NF- κ B activity in the spleen during an infection to ensure the activity of adaptive immune responses, and that this activation of NF- κ B might be through the alternative

NF- κ B pathway which is important in both development and maintenance of the spleen (13).

In the search for mechanisms behind the protective effects of plant-based diets, reductionistic approaches studying isolated compounds have dominated. Such an approach might fit well into pharmacological research, but in doing so, we might overlook additive, synergistic or antagonistic effects that arise from combinations of compounds found in plant foods and diets. Thus, we have chosen to make extracts of foods the focus of this study. In the present work, we found synergistic inhibitory effects on LPS-induced NF- κ B activation with the combination of five dietary plant extracts, as compared to the sum of effects of the five individual extracts. Keeping the concentration for the single extracts constant, the inhibitory synergistic effect on NF- κ B activation by the combined extract persisted even at half the concentration. At a level of 1/5th concentration the added concentration of extracted foods in the combined extract is comparable to each individual extract tested alone, meaning that the effect of the combined extract is not simply an effect of a higher dose of food. This underlines the potential of synergistic effects being created by a variety of foods. Such effects would not be detectable in a more reductionistic model.

The most recent report from the World Cancer Research Fond/American Institute for Cancer Research (19) concludes that coffee is unlikely to have an effect on cancers of the pancreas or kidneys. At present there is limited evidence on effects of coffee intake on other cancers sites, however some epidemiological studies suggest preventive effects of coffee in the liver (37-39). In a large epidemiological study, we observed that intake of coffee was inversely associated with reduced death attributed to oxidative stress and inflammatory diseases (40). Oxidative stress and inflammation are both closely linked to NF- κ B activation and are involved in cancer initiation and development (41;42). In this perspective, inhibition of NF- κ B might contribute to the inverse relationship between coffee intake and disease, with the liver as a main target organ.

Walnuts, clove, thyme and oregano are all rich in redox-active phytochemicals (43;44) however none of these dietary plants have been extensively studied with respect to chemopreventive properties. The observed NF- κ B inhibition by our combination extract may, at least partly, be attributed to rosmarinic acid (45) found in thyme and oregano (46), or eugenol (47) found in clove (46), which are all identified as inhibitors of NF- κ B; however thymol, a major phytochemical in thyme, did not modulate NF- κ B activity (24). Thyme- and oregano essential oils in combination decreased the levels of IL-1 β and IL-6, as well as inflammation related tissue damage in a model of colitis (48), both of which may be a result of NF- κ B inhibition in the colon. Also, thyme may induce the level of endogenous cytoprotective proteins in the liver (49). Walnuts are

also a rich source of essential fatty acids with known anti-inflammatory properties (50).

Approaches that focus on foods rather than single compounds should be valuable in future research to unravel the chemopreventive effects of foods. To our knowledge, this is the first report to indicate synergistic effects of foods on NF- κ B activation, however more research is warranted to confirm and to expand the yet limited knowledge about these synergistic effects. Our experiments show that ordinary dietary plants may be potent modulators of the NF- κ B signaling pathway both *in vitro* and *in vivo*, and thus support a role of chemoprevention through dietary means.

References

- 1 Greten FR, Karin M. The IKK/NF-kappaB activation pathway-a target for prevention and treatment of cancer. *Cancer Lett* 2004 Apr 8;206(2):193-9.
- 2 Luo JL, Maeda S, Hsu LC, Yagita H, Karin M. Inhibition of NF-kappaB in cancer cells converts inflammation-induced tumor growth mediated by TNFalpha to TRAIL-mediated tumor regression. *Cancer Cell* 2004 Sep;6(3):297-305.
- 3 Pikarsky E, Porat RM, Stein I, *et al.* NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004 Sep 23;431(7007):461-6.
- 4 Karin M, Yamamoto Y, Wang QM. The IKK NF-kappaB system: a treasure trove for drug development. *Nat Rev Drug Discov* 2004 Jan;3(1):17-26.
- 5 Valen G, Yan ZQ, Hansson GK. Nuclear factor kappa-B and the heart. *J Am Coll Cardiol* 2001 Aug;38(2):307-14.
- 6 Mattson MP, Camandola S. NF-kappaB in neuronal plasticity and neurodegenerative disorders. *J Clin Invest* 2001 Feb;107(3):247-54.
- 7 Feldmann M, Andreakos E, Smith C, *et al.* Is NF-kappaB a useful therapeutic target in rheumatoid arthritis? *Ann Rheum Dis* 2002 Nov;61 Suppl 2:ii13-ii18.
- 8 Christman JW, Sadikot RT, Blackwell TS. The role of nuclear factor-kappa B in pulmonary diseases. *Chest* 2000 May;117(5):1482-7.
- 9 Neurath MF, Becker C, Barbulescu K. Role of NF-kappaB in immune and inflammatory

- responses in the gut. *Gut* 1998 Dec;43(6):856-60.
- 10 Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2002 Apr;2(4):301-10.
 - 11 Colombo MP, Mantovani A. Targeting myelomonocytic cells to revert inflammation-dependent cancer promotion. *Cancer Res* 2005 Oct 15;65(20):9113-6.
 - 12 Maeda S, Kamata H, Luo JL, Leffert H, Karin M. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell* 2005 Jul 1;121(7):977-90.
 - 13 Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* 2004 Jun;25(6):280-8.
 - 14 Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 1999 Nov 22;18(49):6853-66.
 - 15 Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 2005 Oct;5(10):749-59.
 - 16 Surh YJ. Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* 2003 Oct;3(10):768-80.
 - 17 Nakanishi C, Toi M. Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. *Nat Rev Cancer* 2005 Apr;5(4):297-309.
 - 18 Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 2004 May;79(5):727-47.
 - 19 World Cancer Research Fund / American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Washington DC: AICR; 2007.
 - 20 FAO/WHO. Joint FAO/WHO Expert Report on Diet, Nutrition and the Prevention of Chronic Disease. WHO Technical Report Series; 2003. Report No.: 916.
 - 21 Bjelakovic G, Nikolova D, Simonetti RG, Gluud C. Antioxidant supplements for prevention of gastrointestinal cancers: a systematic review and meta-analysis. *Lancet* 2004 Oct 2;364(9441):1219-28.
 - 22 Miller ER, III, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med* 2005 Jan 4;142(1):37-46.
 - 23 Jacobs DR, Jr., Tapsell LC. Food, not nutrients, is the fundamental unit in nutrition. *Nutr Rev* 2007 Oct;65(10):439-50.
 - 24 Paur I, Austenaa LM, Blomhoff R. Extracts of dietary plants are efficient modulators of nuclear factor kappa B. *Food Chem Toxicol* 2008 Apr;46(4):1288-97.
 - 25 Carlsen H, Moskaug JO, Fromm SH, Blomhoff R. In vivo imaging of NF-kappa B activity. *J Immunol* 2002 Feb 1;168(3):1441-6.
 - 26 Park JM, Greten FR, Wong A, *et al.* Signaling pathways and genes that inhibit pathogen-induced macrophage apoptosis--CREB and NF-kappaB as key regulators. *Immunity* 2005 Sep;23(3):319-29.
 - 27 Suganuma M, Okabe S, Oniyama M, Tada Y, Ito H, Fujiki H. Wide distribution of [3H](-)-epigallocatechin gallate, a cancer preventive tea polyphenol, in mouse tissue. *Carcinogenesis* 1998 Oct;19(10):1771-6.
 - 28 Mullen W, Graf BA, Caldwell ST, *et al.* Determination of flavonol metabolites in plasma and tissues of rats by HPLC-radiocounting and tandem mass spectrometry following oral ingestion of [2-(14)C]quercetin-4'-glucoside. *J Agric Food Chem* 2002 Nov 6;50(23):6902-9.
 - 29 Vitrac X, Desmouliere A, Brouillaud B, *et al.* Distribution of [14C]-trans-resveratrol, a cancer chemopreventive polyphenol, in mouse tissues after oral administration. *Life Sci* 2003 Apr 4;72(20):2219-33.
 - 30 Liu GH, Qu J, Shen X. NF-kappaB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochim Biophys Acta* 2008 May;1783(5):713-27.
 - 31 Li W, Khor TO, Xu C, *et al.* Activation of Nrf2-antioxidant signaling attenuates NFkappaB-inflammatory response and elicits apoptosis. *Biochem Pharmacol* 2008 Jul 23.
 - 32 Surh YJ, Na HK. NF-kappaB and Nrf2 as prime molecular targets for chemoprevention and cytoprotection with anti-inflammatory

- and antioxidant phytochemicals. *Genes Nutr* 2008 Feb;2(4):313-7.
- 33 Dinkova-Kostova AT, Talalay P. Direct and indirect antioxidant properties of inducers of cytoprotective proteins. *Mol Nutr Food Res* 2008 Jun;52 Suppl 1:S128-S138.
 - 34 Ramos-Gomez M, Kwak MK, Dolan PM, *et al.* Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proc Natl Acad Sci U S A* 2001 Mar 13;98(6):3410-5.
 - 35 Khor TO, Huang MT, Prawan A, *et al.* Increased Susceptibility of Nrf2 Knockout Mice to Colitis-Associated Colorectal Cancer. *Cancer Prevention Research* 2008 Aug 1;1(3):187-91.
 - 36 Xu W, Hellerbrand C, Kohler UA, *et al.* The Nrf2 transcription factor protects from toxin-induced liver injury and fibrosis. *Lab Invest* 2008 Oct;88(10):1068-78.
 - 37 Ruhl CE, Everhart JE. Coffee and tea consumption are associated with a lower incidence of chronic liver disease in the United States. *Gastroenterology* 2005 Dec;129(6):1928-36.
 - 38 Larsson SC, Wolk A. Coffee consumption and risk of liver cancer: a meta-analysis. *Gastroenterology* 2007 May;132(5):1740-5.
 - 39 Wakai K, Kurozawa Y, Shibata A, *et al.* Liver cancer risk, coffee, and hepatitis C virus infection: a nested case-control study in Japan. *Br J Cancer* 2007 Aug 6;97(3):426-8.
 - 40 Andersen LF, Jacobs DR, Jr., Carlsen MH, Blomhoff R. Consumption of coffee is associated with reduced risk of death attributed to inflammatory and cardiovascular diseases in the Iowa Women's Health Study. *Am J Clin Nutr* 2006 May;83(5):1039-46.
 - 41 Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008 Jul 24;454(7203):436-44.
 - 42 Hussain SP, Hofseth LJ, Harris CC. Radical causes of cancer. *Nat Rev Cancer* 2003 Apr;3(4):276-85.
 - 43 Dragland S, Senoo H, Wake K, Holte K, Blomhoff R. Several Culinary and Medicinal Herbs Are Important Sources of Dietary Antioxidants. *J Nutr* 2003 May;133(5):1286-90.
 - 44 Halvorsen BL, Holte K, Myhrstad MC, *et al.* A systematic screening of total antioxidants in dietary plants. *J Nutr* 2002 Mar;132(3):461-71.
 - 45 Lee J, Jung E, Kim Y, *et al.* Rosmarinic acid as a downstream inhibitor of IKK-beta in TNF-alpha-induced upregulation of CCL11 and CCR3. *Br J Pharmacol* 2006 Jun;148(3):366-75.
 - 46 Shan B, Cai YZ, Sun M, Corke H. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *J Agric Food Chem* 2005 Oct 5;53(20):7749-59.
 - 47 Chainy GB, Manna SK, Chaturvedi MM, Aggarwal BB. Anethole blocks both early and late cellular responses transduced by tumor necrosis factor: effect on NF-kappaB, AP-1, JNK, MAPKK and apoptosis. *Oncogene* 2000 Jun 8;19(25):2943-50.
 - 48 Bukovska A, Cikos S, Juhas S, Il'kova G, Rehak P, Koppel J. Effects of a combination of thyme and oregano essential oils on TNBS-induced colitis in mice. *Mediators Inflamm* 2007;2007:23296.
 - 49 Sasaki K, Wada K, Tanaka Y, Yoshimura T, Matuoka K, Anno T. Thyme (*Thymus vulgaris* L.) leaves and its constituents increase the activities of xenobiotic-metabolizing enzymes in mouse liver. *J Med Food* 2005;8(2):184-9.
 - 50 Ros E, Mataix J. Fatty acid composition of nuts--implications for cardiovascular health. *Br J Nutr* 2006 Nov;96 Suppl 2:S29-S35.

Tables

Table 1 Assessment of synergy: difference in $\ln(\text{NF-}\kappa\text{B})$ according to prediction from main effects of the 5 ingredients in the combined extract compared to the effect of the combined extract

Main effects				
Parameter	Estimate	SE	t Value	Pr > t
A)				
Combined extract at 3/0.3 mg/ml* versus sum of 5 at 3/0.3 mg/ml				
Sum of 5 ingredients	-0.42	0.21	-1.95	0.075
Combined extract	-2.14	0.23	-9.17	<.001
Difference Combined extract versus Sum of 5	-1.72	0.44	-3.93	0.002
B)				
Combined extract at 1.5/0.15 mg/ml* versus sum of 5 at 3/0.3 mg/ml				
Sum of 5 ingredients	-0.42	0.24	-1.71	0.112
Combined extract	-1.99	0.27	-7.49	<.001
Difference Combined extract versus Sum of 5	-1.57	0.50	-3.15	0.008
C)				
Combined extract at 0.6/0.06 mg/ml* versus sum of 5 at 3/0.3 mg/ml				
Sum of 5 ingredients	-0.42	0.20	-2.12	0.056
Combined extract	-0.26	0.21	-1.23	0.242
Difference Combined extract versus Sum of 5	0.15	0.40	0.38	0.711

* Concentrations: a/b mg/ml = a mg/ml of each of coffee and walnut and b mg/ml of each of clove, thyme and oregano

Figure Legends

Figure 1 Dietary Plant Extracts inhibit NF- κ B activation *in vitro*

U937- κ B cells were incubated with the indicated extract for 6.5h, and LPS was added for the last 6h. Luciferase activity was measured by imaging. Bars represent mean of 2 or 3 experiments each performed in triplicate \pm SEM. * = $p < 0.05$. The image of one representative experiment is shown in the lower half of the figure. Luminescence was measured in photons/sec/cm²/steradian, and presented with the color bar. Gray scale images were used for reference of position. A) Bars indicate fold change in luminescence at the indicated dosage of the combined extract. B) Bars indicate fold change in luminescence at the indicated dosage of LPS or of LPS plus the single extract.

Figure 2 Combination extract inhibits NF- κ B *in vivo*

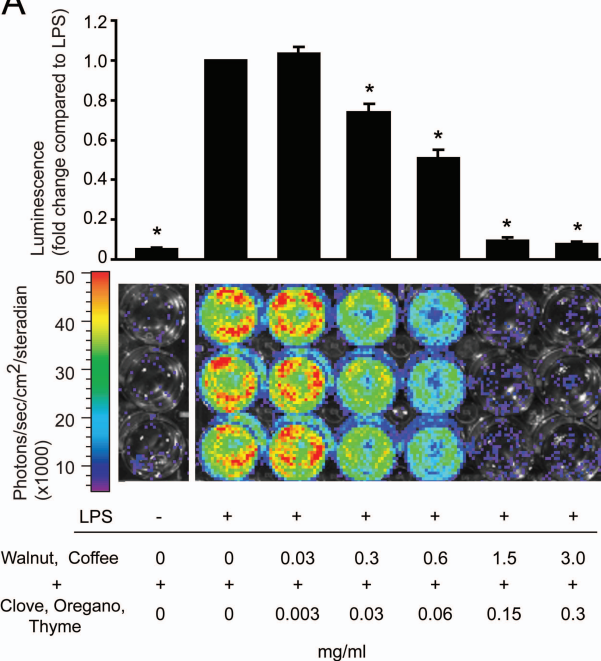
Transgenic NF- κ B-luciferase reporter mice were given a single dose of extract by oral gavage 3h prior to s.c. LPS injection (at 0h). A) The luciferase activity was measured by *in vivo* imaging at 0h, 2h, 4h and 6h (one representative mouse from each group is shown). B) The average fold change (\pm SEM) of emittance of photons/sec/cm²/steradian (normalized to 0h) at 0h, 2h, 4h and 6h from the whole mice minus the head, limbs and tail (LPS + extract = open squares, LPS only = closed circles) (n = 16). C) The AUC was calculated for each mouse and compared between the two treatment groups. The average photons emitted and AUC were compared using the Student's t-test. * = $p < 0.05$.

Figure 3 Combination extract inhibits NF- κ B activation in intestine, liver, testis and epididymis

Directly following imaging at 6h the mice were euthanized, and organs excised. A) *Ex vivo* imaging of the intestines was performed. The average fold change (\pm SEM) of luciferase activity from the intestines of LPS-only mice (grey bars) versus LPS + Extract mice (black bars) is shown. B) Image of one representative intestine of a mouse from each treatment group is shown. Luminescence was measured in photons/sec/cm²/steradian, and presented with the color bar. Gray scale images were used for reference of position. C) Luciferase activity was measured in tissue homogenates and normalized to the average of LPS-only mice. Mean \pm SEM. WAT = White Adipose Tissue. Student's t-tests were used to compare the two treatments. n = 16 for all organs except uterus n = 10, testis & epididymis n = 6. * = $p < 0.05$

Figure 1, Paur et al.

A



B

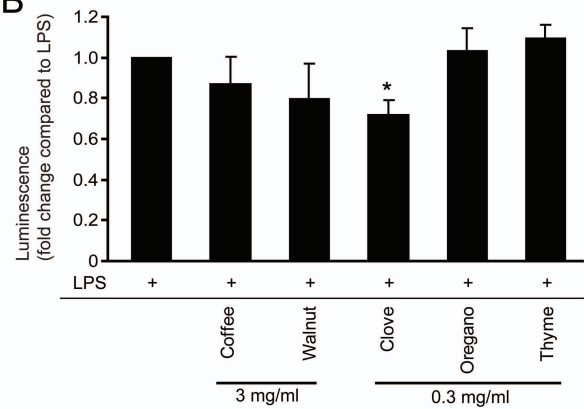


Figure 2, Paur et al.

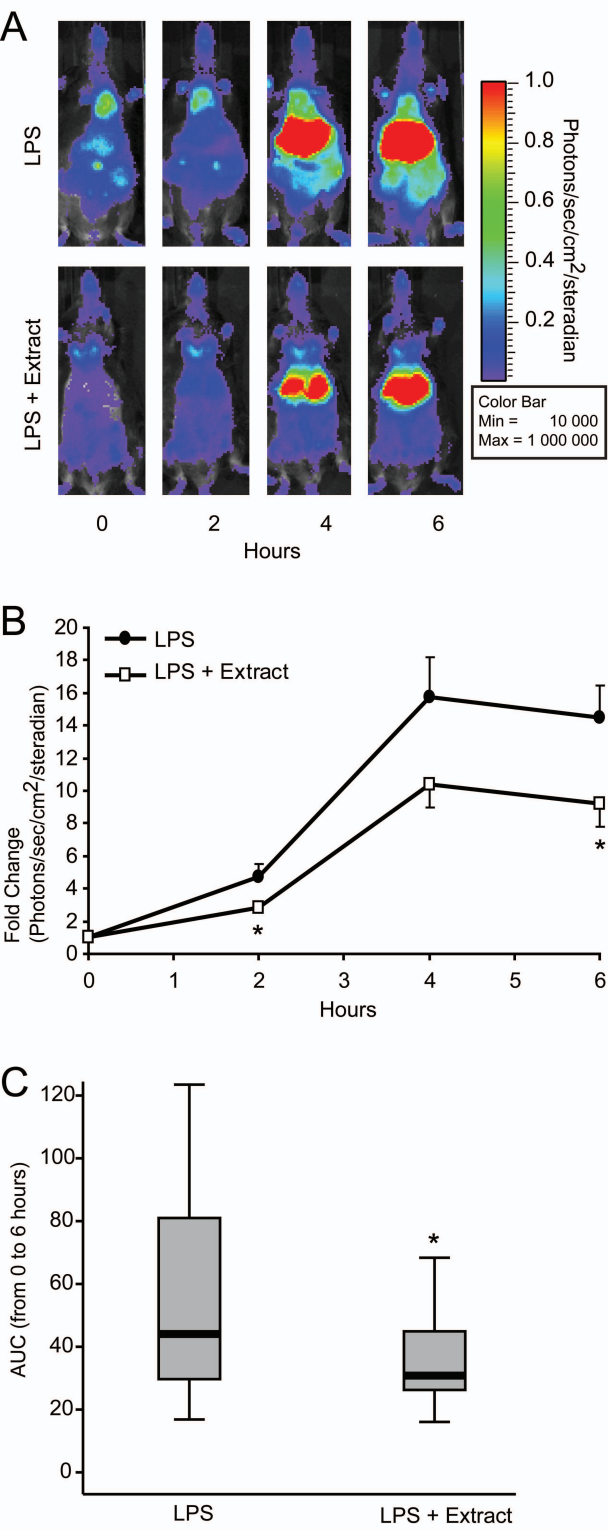


Figure 3, Paur et al.

